

EFFECTS OF INTESTINAL ISCHAEMIA AND
REPERFUSION ON THE GUT AND LIVER

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Abstract

Intestinal ischaemia-reperfusion (IIR) injury is a life-threatening condition affecting adults and children. Common causes include acute mesenteric ischaemia, volvulus, intussusception, necrotizing enterocolitis, sepsis and trauma. IIR triggers a cascade of inflammatory mediators, increases intestinal permeability and results in local and remote organ damage. The liver is especially prone to IIR-associated dysfunction. Adults and children respond to IIR injury in subtly different ways. This thesis reports an investigation of a rat model of IIR (90 minutes intestinal ischaemia then 60 minutes reperfusion under general anaesthetic) and describes IIR-related changes in the liver, blood composition and gut. Hepatic tissue metabolites were measured after IIR in suckling and adult rats. In both age groups ATP fell and inorganic phosphate (Pi) and alanine rose after IIR. Adult rats had higher levels of total hepatic glutathione, and hepatic glutamine fell after IIR. Hepatic lactate and succinate rose in suckling rats after IIR. Blood composition changed after IIR in adult rats – Pi rose, haematocrit rose, pH fell, and blood oxygen content rose. Systemic, but not portal, arterio-venous difference in oxygen content rose. Hepatic inflow fell with prolonged intestinal reperfusion, which was partially abrogated by whole-body hypothermia ($32 \pm 0.5^{\circ}\text{C}$). Hypothermia also reduced IIR-related changes in blood composition and hepatic tissue metabolites, abolishing the relationship of the latter with final total hepatic inflow. IIR was associated with rises in plasma tumour necrosis factor- α , interleukins 6 and 10, and endotoxin. These rises were significantly reduced by hypothermia. Correlation of intestinal arterial inflow and intestinal tissue oxygenation index during IIR demonstrated a complex relationship. In conclusion, IIR perturbs hepatic metabolism, blood composition, hepatic inflow, and plasma cytokine and endotoxin levels. Some of these effects are abrogated by hypothermia. It is possible to follow intestinal tissue oxygenation during IIR with near infra-red spectroscopy. These latter observations may have clinical significance.

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List of Abbreviations

AAA	abdominal aortic aneurysm
ADP	adenosine diphosphate
αGST	alpha-glutathione S-transferase
ALT	alanine transaminase
AMP	adenosine monophosphate
AP-1	activator protein-1
APACHE	acute physiology and chronic health evaluation
ARDS	acute respiratory distress syndrome
AST	aspartate transaminase
ATP	adenosine triphosphate
A-V	arterio-venous
C3 _a	complement fragment 3 _a
C4 _a	complement fragment 4 _a
C5 _a	complement fragment 5 _a
CD4	cluster determinant 4
CD11	cluster determinant 11
CD18	cluster determinant 18
CINC	cytokine-induced neutrophil chemoattractant
cNOS	constitutive nitric oxide synthetase
CT	computed tomography
DHCA	deep hypothermic circulatory arrest
DNA	deoxyribonucleic acid
E. Coli	Escherichia coli
ED-1	monocyte/macrophage marker, clone ED-1
ED-2	monocyte/macrophage marker, clone ED-2
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
eNOS	endothelial cell nitric oxide synthetase
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony stimulating factor
GMP	guanosine monophosphate
HAF	hepatic arterial flow
Hb	deoxyhaemoglobin
HbO	oxyhaemoglobin
HPLC	high performance liquid chromatography
HSF	heat shock factor
HSP	heat shock protein
HSR	heat shock response
ICAM-1	intercellular adhesion molecule-1
ICAM-2	intercellular adhesion molecule-2
ICAM-3	intercellular adhesion molecule-3

ICU	intensive care unit
IFN- γ	interferon-gamma
IIR	intestinal ischaemia-reperfusion
I κ B α	inhibitory protein κ B α
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IO	intestinal ischaemia only
iNOS	inducible nitric oxide synthetase
I/R	ischaemia-reperfusion
IVC	inferior vena cava
LFA-1/CD11A/CD18	lymphocyte function-associated antigen-1
L-NMMA	NG-monomethyl-L-arginine
LPS	lipopolysaccharide
LTB $_4$	leukotriene B $_4$
Mac-1/CD11B/CD18	macrophage antigen-1
MAP	mean arterial blood pressure
MBF	mesenteric blood flow
MCP-1	monocyte chemoattractant protein-1
MDA	malonyldialdehyde
MDP	methylenediphosphonic acid
MGV	mid-gut volvulus
MIP	macrophage inflammatory protein
MMP	mitochondrial membrane potential
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRS	magnetic resonance spectroscopy
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NEC	necrotizing enterocolitis
NF κ B	nuclear factor κ B
NICU	neonatal intensive care unit
NIRS	near infra-red spectroscopy
nNOS	neuronal cell nitric oxide synthetase
NO	nitric oxide
NOS	nitric oxide synthetase
ONOO \cdot	peroxynitrite
PAF	platelet activating factor
pHi	intramucosal pH
Pi	inorganic phosphate
PMNs	polymorphonuclear leukocytes/neutrophils
pO $_2$	partial pressure of oxygen

PVF	portal venous flow
RA	right atrium
RANTES.....	regulated on activation normal T-cell
.....	expressed and secreted molecule
RNA.....	ribonucleic acid
RNOS	reactive nitrogen oxygen species
ROI.....	remote organ injury
ROS.....	reactive oxygen species
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
SMA.....	superior mesenteric artery/arteria
SMAF	superior mesenteric arterial flow
SMV	superior mesenteric vein/venous
TBARS.....	thiobarbituric acid reactive substances
THI.....	total hepatic inflow
TIPS	transjugular intrahepatic portosystemic shunt
TNF- α	tumour necrosis factor-alpha
TNFR.....	tumour necrosis factor-alpha receptor
TOI.....	tissue oxygenation index
TPN.....	total parenteral nutrition
TRADD	tumour necrosis factor-alpha receptor-associated
.....	death domain protein
TSP.....	sodium 3-trimehtlysilyl-[2,2,3,3,-d ₄]-propionate
TTUS.....	transit-time ultrasound
VCAM	vascular cell adhesion molecule

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Chapter 1: Introduction to intestinal ischaemia-reperfusion

Introduction

Intestinal ischaemia-reperfusion (IIR) injury is a major cause of morbidity and mortality in both adults and children. IIR may be a consequence of several clinical conditions including acute or chronic mesenteric ischaemia, volvulus, strangulated intestinal hernia, intussusception, necrotizing enterocolitis, severe sepsis, burns, haemorrhagic shock, surgery and portal or hepatic pathology. IIR triggers a cascade of molecular, cellular and whole organ changes in biochemistry and physiology leading to tissue damage and organ dysfunction both locally and systemically. Research has suggested a role for the heat shock response and hypothermia in moderating the effects of IIR. Patient and experimental animal age have been shown to alter the response to IIR both locally and systemically.

This thesis uses a rat model to explore some of the local and remote effects of IIR. The purpose of this introductory chapter is to provide an overview of the anatomy of the splanchnic circulation, common causes and pathophysiology of IIR, and some novel approaches to therapy for IIR injury in order to establish a context within which the work described in later chapters may be placed.

Anatomy

Normal splanchnic circulation

The splanchnic circulation serves organs contained within the abdomen and pelvis (lower oesophagus, stomach, duodenum, jejunum, ileum, caecum, appendix, colon, rectum, pancreas, spleen, gallbladder and biliary ducts, liver and part of the diaphragm).

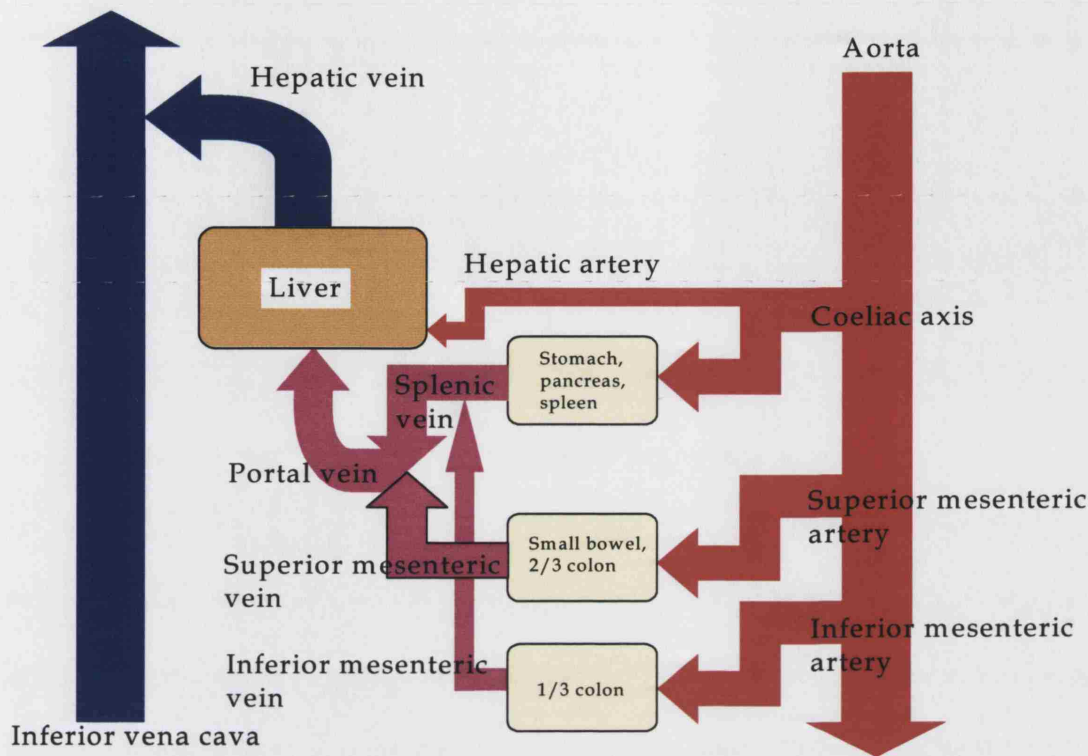


Figure 1.1 Schematic diagram of the splanchnic circulation.

Figure 1.1 shows that the intestines and liver are connected in series between arterial inflow and systemic venous outflow: arterial supply is via the coeliac, superior and inferior mesenteric arteries, and venous drainage is via the portal and hepatic veins. Lymph is collected into intra-abdominal (mainly mesenteric) lymphatics, and re-enters the systemic circulation via the thoracic duct - thus by-passing the portal circulation and filtration/detoxification in the liver. The liver has a dual blood supply via the hepatic artery (carrying systemic arterial blood) and the portal vein (carrying venous efflux from the intestines, pancreas and spleen). The hepatic artery contributes 20-30% of total hepatic inflow (Jiao et al, 2000; Clemmesen et al, 1999) and fifty percent of hepatic oxygen supply: this division is autoregulated locally. Interruption of arterial inflow into and/or venous efflux out of the intestines reduces hepatic inflow via a reduction in portal venous flow. If portal venous flow decreases hepatic arterial flow increases under normal

circumstances. This 'hepatic arterial buffer response' is due to a reduction in clearance of adenosine within the liver when portal venous flow is reduced (Lautt et al, 1985). If the local hepatic paracrine milieu is not normal this reflex may not be observed (Tadros et al, 2000). Portal inflow pressure is also important in passively distending hepatic sinusoids (McCuskey, 2000) and maintaining the vascular 'waterfall', and the passively distensible 'sink' arrangements of the hepatic vasculature (Brienza et al, 1995). Reduced hepatic compliance increases hepatic hydrostatic back-pressure and decreases portal venous inflow (Lautt and Legare, 1992).

Splanchnic organs hold 20-25% of total blood volume (Pastores et al, 1996) and receive 25% of cardiac output (approximately 1.5 litres per minute in humans) at rest. Half of splanchnic blood is in the intestinal circulation (lower oesophagus to rectum), with the remainder lying predominantly in the liver and spleen. Of this intestinal circulating volume, 70% lies in the mucosa and sub-mucosa; the remaining 30% in the muscularis mucosa and serosa. The liver receives 100ml/100g tissue/minute and the intestines 50 – 70ml/100g tissue/minute at rest. The percentage of total blood volume and of cardiac output within the splanchnic circulation rises after meals and falls during exercise and other physical stress. The physiological fall in splanchnic perfusion during exercise and other physically stressful occasions occurs as part of the 'diving reflex' (Scholander, 1963).

Splanchnic oxygen consumption is approximately 50-60 millilitres of oxygen per minute at rest in adults - this represents an extraction efficiency of 15-20%. Absolute oxygen consumption rises during digestion (splanchnic blood flow increases and splanchnic organs actively absorb and assimilate nutrients)(Stevenson and Weiss, 1988). If splanchnic blood flow is reduced by a small amount, absolute oxygen consumption is maintained by more efficient oxygen extraction (Samsel and Schumacker, 1994) – this is termed non-critically low flow. Absolute oxygen consumption eventually falls if blood flow to the splanchnic bed is severely reduced: when blood flow is less than the critical flow

Chapter 1: Introduction to intestinal ischaemia-reperfusion

rate at which oxygen extraction is maximal, absolute oxygen consumption becomes flow dependent, and absolute oxygen consumption falls if inflow is further reduced – this is critically low-flow. The critically low-flow threshold of adult human intestine is 30ml/minute/100g (Desai et al, 1996), resulting in a maximal oxygen extraction of 1.6 ± 0.06 ml O₂/minute/100 g.

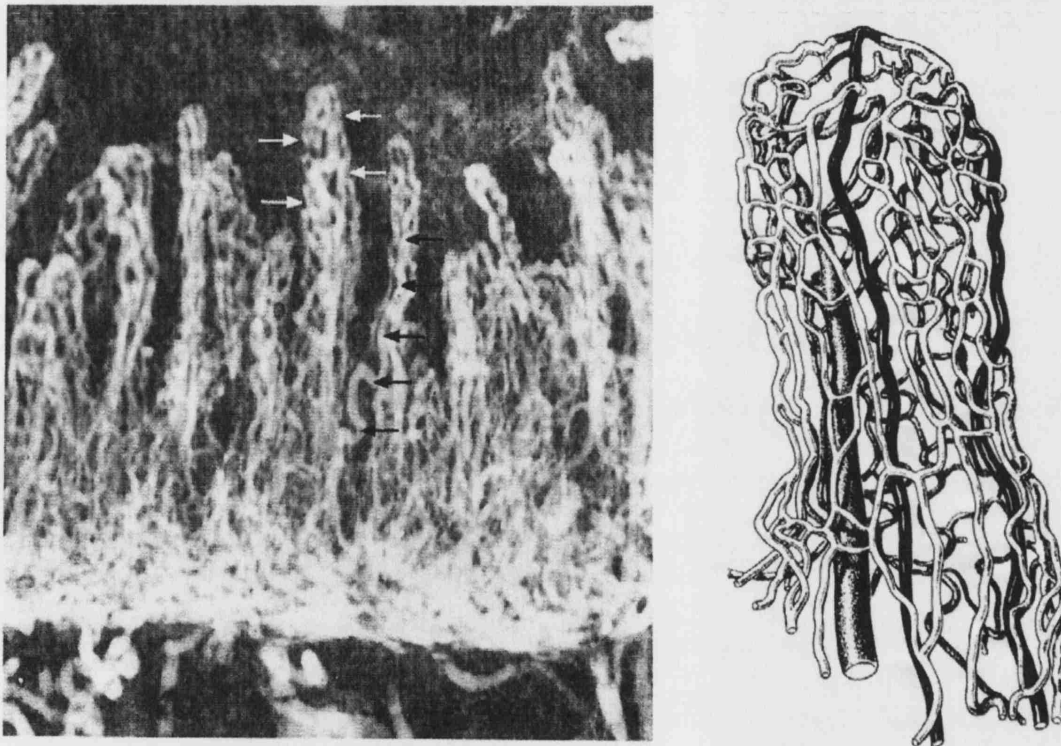


Figure 1.2 Photograph of a radiograph demonstrating the central villous arteriole (black arrows) and surrounding villous venous plexus (white arrows) with an expanded diagram of a single villous [both images reproduced from (Marston et al, 1989)].

Figure 1.2 demonstrates the central arteriole and surrounding venous plexus within the villous. The intestine has a unique distribution of blood vessels within the villous which is thought to make the mucosa particularly vulnerable to ischaemic damage. A 'counter-current' mechanism allows oxygen to diffuse directly from the central arteriole into the surrounding venous plexus, by-passing the villous apical capillary network. At rest oxygen tension in the venous plexus is relatively high (haemoglobin is approximately eighty-five percent saturated in the portal vein at rest) so little oxygen is transferred in this way. If portal venous

Chapter 1: Introduction to intestinal ischaemia-reperfusion

oxygen tension falls a greater proportion of oxygen carried in the central arteriole will diffuse directly into the venous plexus. A positive feedback situation ensues in which progressively less oxygen is delivered to the capillary bed leading to hypoxia in the capillary bed, increased efficiency of capillary oxygen extraction and further reducing oxygen tension in the villous venous plexus. More oxygen will diffuse directly into the venous plexus within the body of the villous and therefore progressively less oxygen will be delivered to the capillary bed. Tissue oxygen tension at the tip of the villous will continue to fall but portal venous oxygen tension will be maintained. If this arterio-venous 'short-circuiting' of oxygen is prolonged, cell death (both by apoptosis and necrosis) and loss of enterocytes at the villous tip will occur. This mechanism demonstrates the unique vulnerability of enterocytes to ischaemic damage. It is not clear whether preservation of portal venous oxygen tension and therefore oxygen supply to the liver over enterocyte survival has an evolutionary advantage, but it may reflect the efficiency with which enterocytes regenerate after ischaemic injury compared to post-ischaemic regeneration of hepatocytes. Mesenteric arteriolar wall (smooth muscle and endothelial cells) oxygen consumption is also significant in the rat (Tsai et al, 1998). This has implications for assumptions of oxygen delivery to the counter-current mechanism in the intestinal villous, and may paradoxically limit the passage of excessive oxygen to the portal vein and support oxygen delivery to the villous tip.

Causes of intestinal ischaemia-reperfusion injury

Causes of IIR injury may be considered in two broad groups: direct - primarily affecting the splanchnic vasculature - and indirect - affecting the entire circulation.

Direct IIR may be purely arterial (mesenteric thromboembolic occlusion) or venous (portal vein thrombosis) or combined (volvulus or intussusception).

Indirect causes of IIR include sepsis, burns and haemorrhagic shock. IIR may be

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observed directly or inferred from intestinal tissue injury or functional deficit.

During global low-flow, organ effects are governed by the 'diving reflex': blood is shunted away from splanchnic organs and skin and preferentially supplies the heart, brain and muscles resulting in an inevitable degree of IIR if flow is restored.

Age, pre-existing morbidity and genetic susceptibility determine which causes of IIR predominate, and influence outcome. Adults develop mesenteric thromboembolus and intestinal obstruction; infants and children develop intussusception, midgut volvulus and necrotizing enterocolitis (NEC). Adults are more likely to be affected by increased pressure within the portal venous system (alcoholic, autoimmune or viral liver disease) or mechanical obstruction (local tumour growth). Global critically low-flow due to sepsis, burns, haemorrhage, cardiovascular insufficiency and surgical or traumatic interruption of flow affect all age groups.

Pre-existing morbidity increases the probability of developing conditions characterised by IIR and the pathological impact of IIR injury. Infants with congenital heart disease suffer a higher incidence of NEC; adults with ischaemic heart disease are more likely to develop atrial fibrillation leading to intra-cardiac blood clot and mesenteric embolic occlusion or significant cardiovascular insufficiency leading to indirect IIR. Alcoholic liver disease reduces physiological reserve to withstand IIR-induced damage and progresses to liver cirrhosis increasing back-pressure in the portal vein causing intestinal venous congestion.

Genetic predisposition to IIR injury is starting to be characterised. Studies on NEC reveal that this condition is associated with increased or reduced levels of several different growth factors. For example, knockout mice lacking epidermal growth factor spontaneously develop NEC-like haemorrhagic enteritis (Shin et al, 2000). This implies that variation in expression or reception of this growth factor may help to explain the sporadic incidence of NEC. Tumour necrosis factor- α (TNF- α) gene promoter polymorphism has been linked to group differences in clinical outcome in surgical patients and septic patients (Fijen et al, 2001).

Unfortunately results are not consistent across studies and prospective data collection is still ongoing.

Direct intestinal ischaemia-reperfusion injury

Mesenteric ischaemia

Acute mesenteric ischaemia has a high morbidity and mortality rate (Jamieson, 1988; Clavien et al, 1987; Gorey and Sullivan, 1988; Wilson et al, 1987). The length of bowel infarcted is influenced by the cause of mesenteric ischaemia: arterial thrombus typically occludes the superior mesenteric artery at its origin from the aorta infarcting the whole mid-gut [from the third part of the duodenum to the splenic flexure] – this accounts for 39% of cases (Gorey and Sullivan, 1988); whereas arterial embolus typically occludes the superior mesenteric artery beyond the proximal jejunal and middle colic arteries allowing the survival of the upper jejunum and right colon – this accounts for 18% of cases (Gorey and Sullivan, 1988). Emboli are most commonly mural thrombi from the auricle of the left atrium developed during periods of atrial fibrillation (90-95% of cases). Venous thrombosis accounts for less than 10% of cases and causes include hypercoagulable states (dehydration, ascending infection, polycythemia), direct compression of the portal vein (neoplastic growths, intestinal bands) and drug use. Chronic intestinal ischaemia becomes symptomatic only after two out of three splanchnic vessels occlude and the third stenoses. Gradual loss of arterial supply allows collaterals to develop and intestinal blood supply is maintained. Non-occlusive mesenteric ischaemia arises from progressive loss of inflow to the splanchnic organs thought to be due to excessive vasoconstriction and is more difficult to treat (Lock and Scholmerich, 1995; Wilcox et al, 1995). Peak mortality for men is at age 70 and for women at age 85 – this may reflect the relative incidence of cardiovascular disease in the sexes. It is possible that routine anticoagulation for atrial fibrillation and widespread use of cholesterol-lowering medication may decrease the incidence of this condition.

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Diagnosis of mesenteric ischaemia is clinical (sudden onset of severe abdominal pain out of all proportion to physical signs for acute ischaemia, and weight loss due to food aversion caused by abdominal pain after eating ['mesenteric angina'] for chronic ischaemia), and radiological (mesenteric angiography demonstrates blockage of the mesenteric arterial inflow either at the origin of the splanchnic arteries from the aorta [acute mesenteric thrombus or atherosclerotic occlusion] or in the superior mesenteric artery after the proximal jejunal and middle colic arteries have been given off [acute mesenteric embolus], or failure of mesenteric venous efflux; contrast enhanced computed tomography [CT] scanning demonstrates failure of contrast enhancement of the intestines), but may be delayed until exploratory laparotomy has been undertaken due to the paucity of specific symptoms and signs associated with this condition.

Treatment may be operative or radiological and is aimed at restoring the blood supply to the affected bowel. Radiologically positioned, catheter-delivered thrombolysis dissolves arterial thrombi and possibly emboli; vasodilators for non-occlusive mesenteric ischaemia improve local blood flow. Repeated arteriograms are required to follow progress and surgery may still be required to resect bowel that does not reperfuse. Surgery may attempt revascularisation of the intestines (Bjorck et al, 2002), and necrotic gut is resected and the ends exteriorised until the extent of intestinal loss is clear. If the intestines are of doubtful viability, the abdomen is closed and a 'second-look' laparotomy performed after 12-24 hours. If the superior mesenteric artery had been occluded long enough to make the whole of the mid-gut necrotic, the surviving patient will be dependent on total parenteral nutrition (TPN) for the remainder of life unless they have a successful small bowel transplant. This is not deemed to be an acceptable quality of life for an elderly patient even if surgery was survived, and in these cases surgical intervention is not pursued. Younger patients may have the majority of their mid-gut resected and be placed on an intestinal failure programme if they recover. Revascularisation procedures for chronic mesenteric ischaemia are associated with the multiple

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organ dysfunction syndrome (MODS) (Harward et al, 1993). Post-operative anticoagulation is necessary in patients suffering embolus to reduce the risk of further embolization (Blackbourne, 1998; Halloran and Baxter, 1996; Mason and Fielding, 1998).

Intestinal obstruction - adults

Intestinal obstruction is a very common cause of intestinal ischaemia and reperfusion injury, but the true incidence is difficult to determine as many episodes are sub-clinical (i.e. do not result in patients seeking medical attention). Causes include intraluminal obstruction (tumour or bolus) or extraluminal compression (volvulus, strangulation or adhesions) of the bowel.

Small bowel obstruction due to post-operative or post-peritonitic adhesions has overtaken strangulated external hernia (part of the bowel is trapped outside of its normal body cavity and undergoes vascular compromise) as the commonest cause in Western Europe and the USA since adoption of elective repair of external hernias. Prior to this strangulated external hernias accounted for almost 50% of cases of intestinal obstruction. Occasionally only a knuckle of bowel is trapped in a hernia causing local IIR, but the lumen of the bowel remains in continuity (Richter's hernia).

Large bowel obstruction is usually secondary to neoplastic tumours in Western Europe and the USA, but in Eastern Europe, Africa and Asia large bowel volvulus is more common. Intestinal regions prone to volvulus (turning on a mesenteric pedicle, cutting off arterial inflow, venous efflux and the lumen of the bowel) are the sigmoid colon, stomach and caecum. Sigmoid volvulus accounts for 4% of all intestinal obstruction in Western Europe and the USA, and is commoner after prolonged aperient use (especially in the elderly and/or psychiatrically disturbed); it is associated with a long, redundant sigmoid colon mobile on a lengthened mesentery. Caecal volvulus accounts for less than 1% of intestinal obstruction (30% of colonic volvuli); it is due to persistence of the caecal mesentery allowing abnormal mobility of the caecum. Gastric volvulus is associated with

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para-oesophageal hiatus hernia and is often asymptomatic (diagnosis being a chance finding on an upper gastrointestinal tract barium study). Clinically it appears that gut predisposed to volvulus volves and de-volves repeatedly and may build up tolerance to IIR injury – this is ischaemic preconditioning (Ishida et al, 1997). This is a difficult area to research but experimental work supports the development of greater tolerance to severe ischaemia-reperfusion (I/R) injury after a series of minor I/R insults in both direct (Miner et al, 1999; Davis et al, 1999; McCallion et al, 2000; Baldwin et al, 2002; Ar et al, 1996) and remote (Gho et al, 1996; Wang et al, 2001; Tang et al, 1999; Xiao et al, 2001; Dickson et al, 2001) cardiac and intestinal injury.

Intestinal obstruction due to intraluminal factors causes IIR because pressure within the bowel lumen increases to the extent that circulation of blood within the bowel wall is prevented, rendering the bowel wall ischaemic. If pressure is relieved, circulation is restored and reperfusion is permitted.

Diagnosis is clinical (abdominal pain, abdominal distension, vomiting, absolute constipation, possibly discolouration of overlying skin with a hernia) and radiological (loops of dilated large or small bowel on plain abdominal X-radiographs). In volvulus, plain X-radiographs may show a dilated loop of large bowel arising from the pelvis forming an omega loop with bird's beak narrowing of the colon indicating the site of the obstruction [sigmoid], or a large gas-filled loop of bowel in the centre of the abdomen and an empty left colon [caecal]. If symptoms, signs and plain X-radiographs are equivocal, barium studies (meal or enema) will demonstrate the pathology.)

Treatment aims to relieve the obstruction - reducing the pressure within the bowel, allowing normal bowel wall circulation and passage of bowel contents - and is expectant, radiological or operative. Expectant treatment (nasogastric drainage, intra-venous fluids, careful fluid and electrolyte balance) is reserved for adhesional obstruction. Radiological relief of large bowel obstruction is achieved by positioning an expanding metal stent within a distal large bowel tumour or by

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untwisting volved bowel using hydrostatic pressure. Operative intervention aims to separate adherent loops of small bowel, resect the obstructed portion of large bowel, untwist volved bowel and anchor it to prevent re-volution or reduce strangulated bowel. Prevention of recurrence of intestinal obstruction due to adhesions has been aided by the introduction of starch-free gloves which has lowered the incidence of post-operative adhesions, and the instillation of Adept® (a 4% solution of icodextrin) (Muller et al, 2003; diZerega et al, 2002) or tissue plasminogen activator (Menzies and Ellis, 1991) into the abdominal cavity on closure, and the use of hyaluronidase (Rodgers et al, 1997) or polyethylene glycol/polylactic acid (Rodgers et al, 1998) gel layers or a Baker tube (a long tube placed within the small bowel to maintain luminal patency during the post-operative period). Any bowel that is frankly necrotic or of dubious viability at operation is resected. Relief of obstruction and restoration of normal intra-luminal pressure precipitates a degree of reperfusion injury. The degree of IIR-related dysfunction both locally and remotely will be proportional to the length of bowel affected and the duration of ischaemia (Paterson-Brown, 1998).

Intussusception

The gut can intussuscept (invaginate within the lumen of the adjacent length of intestine) causing vascular and mechanical obstruction of the bowel. Intussusception affects 4/1000 infants and children per year in the UK and is less common in adults. The commonest site of intussusception is ileocolic, but ileoileocolic, ileoileal and colocolic intussusception are also described (in descending order of frequency). The presence of a polyp, Meckel's diverticulum, ectopic pancreatic tissue, duplication cyst or a stricture are the commonest identified causes of intussusception, but the majority of cases (more than 90%) are impossible to ascribe to a physical abnormality. Most are associated with inflamed Peyer's patches in the ileum and occur after an upper respiratory tract infection.

Symptoms include severe colicky pain and vomiting interspersed with normal health. After 24 hours, a characteristic 'red currant jelly' stool may be

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passed. A sausage-shaped mass may be palpable in the upper abdomen, and rectal examination reveals blood in most cases. Intussusception is associated with release of cytokines, lipopolysaccharide and acute phase proteins (Willetts, 2001). Ultrasound or contrast enema confirms the diagnosis of intussusception. Reduction of the intussusception is attempted by gentle hydrostatic pressure within the lumen and is successful in the majority of cases. If this fails, open reduction is performed and severely damaged gut or a non-reducible intussusception can be resected. Any anatomical lead-point identified is resected to prevent recurrence (approximately 3% recur). Overall mortality from intussusception is low if managed promptly, and is related to delay in diagnosis leading to gangrenous bowel, perforation, peritonitis and severe sepsis.

Intestinal obstruction - neonatal and paediatric

There are many causes of intestinal obstruction predominantly affecting infants and children: for example Hirschsprung's disease (aganglionosis of the distal colon), meconium ileus and mid-gut volvulus (MGV). As MGV is the clearest instance of IIR, I will confine my comments to this condition in this section.

MGV due to intestinal malrotation affects 1/10 000 live births and has an early mortality rate of 20% proportional to the extent of intestinal gangrene found at laparotomy. MGV is associated with anterior abdominal wall defects (exomphalos and gastroschisis) and congenital diaphragmatic hernia. The duodenojejunal junction lies to the right of the midline and the caecum is usually in the upper abdomen. The caecum may give rise to peritoneal attachments that cross the duodenum to attach to the posterior abdominal wall (Ladd's bands). In the presence of a narrow mesenteric base, the mid-gut (the part of the gut supplied by the superior mesenteric artery – the jejunum, ileum, appendix, caecum, ascending colon and transverse colon) can twist on its vascular pedicle, obstructing the lymphatic and venous drainage of the mid-gut. This induces a mixed arterial and venous intestinal ischaemic insult, and can lead to necrosis and loss of the

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whole mid-gut if not swiftly rectified. Mid-gut volvulus can occur without malrotation of the mid-gut but this is extremely rare.

Diagnosis of MGV is made via clinical suspicion (intermittent bile-stained vomiting and pain; a sick child with rectal bleeding and abdominal tenderness are late signs) and x-ray findings (abnormal pattern of bowel gas indicating a dilated duodenum, displaced caecum and small bowel obstruction).

MGV is treated by de-volving the gut mesentery at operation. Restoration of arterial inflow is confirmed and the intestines are assessed for viability. If more than 50 cm of small bowel is clearly viable, necrotic bowel can be resected with a reasonable prospect of functional recovery of the intestines. If only a short length of small bowel appears viable, no resection is performed at the first laparotomy and the intestines are replaced into the abdominal cavity with the small bowel to the right and caecum and ascending colon to the left. This widens and stabilises the mesentery reducing the probability of the volvulus recurring. Due to the abnormal position of the appendix, this is removed to avoid diagnostic confusion later in life. The infant is watched closely in the post-operative period for the development of reperfusion injury. This can be manifest by a marked deterioration in clinical condition with oedema of the bowel \pm perforation, abdominal hypertension, haemodynamic instability, sepsis, respiratory and renal compromise, clotting abnormalities and persistent acidosis. A second look laparotomy is performed to reassess intestinal viability after 24 hours if this was not clear or very little bowel was viable at the original operation. 10% of patients undergo further laparotomy for volvulus recurrence or adhesion-related obstruction (Drake, 1998).

Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is the commonest acquired gastrointestinal emergency in the neonate and affected 1 – 3/1000 live births between 1979 and 1985 in the U.S.A. (Holman et al, 1989). NEC accounted for 13.1 deaths per 100 000 live births in the same period; this represents 1.1% of all infant mortality. In the

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U.K., NEC affects 0.4 – 14/1000 live births and approximately 5% of neonatal intensive care unit (NICU) admissions. About 300 cases of NEC are reported annually, and between 70 and 100 deaths are ascribed to NEC per year in the UK (Morecroft and Coombs, 1997). Severe NEC has a 16-40% acute mortality rate (Kosloske, 1997; Fasoli et al, 1999; Hall et al, 2002; Osborn et al, 1999).

NEC results in a spectrum of disease ranging from mild inflammation of the intestinal mucosa to transmural necrosis, pneumatosis intestinalis, intestinal perforation, sepsis and progressive MODS. The intestinal injuries seen in NEC are histologically similar to those induced by IIR – hence IIR injury may represent a final common pathway for the insult(s) giving rise to the NEC. Despite numerous epidemiological and animal studies, the pathogenesis of NEC has not been satisfactorily described. It is thought to involve a combination of factors including episodes of IIR (via cold stress, shock or hypoxia), abnormal bacterial colonisation of the intestine, and the presence of a substrate conducive to intestinal damage within the gut lumen (Santulli et al, 1975). There is likely to be an element of host susceptibility because 95% of infants with risk factors identical to infants developing NEC do not develop NEC (Kosloske, 1990). Clusters of NEC are intermittently reported by NICUs leading to the suspicion of an infective cause, but no one organism has been convincingly associated with these outbreaks. NICU babies have a higher incidence of NEC (1 – 7.7% of those admitted to NICUs) which may be due to the higher prevalence of prematurity, sepsis and co-morbidity in this group. Congenital cardiovascular disease also carries a higher risk of NEC – up to 3.3% with complex malformations (McElhinney et al, 2000). The majority of babies with congenital cardiovascular disease who develop NEC are full-term infants. They develop NEC following cardiac surgery and therefore may be a global low-flow induced NEC: this may indicate distinct sub-pathology within this patient group because full-term infants normally represent only 10% of NEC patients.

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Diagnosis of NEC is clinical as no specific diagnostic tests are available to date. Neonates develop bloody diarrhoea, abdominal distension and bilious vomiting. In severe cases the abdominal wall may become erythematous, which implies full-thickness intestinal wall necrosis. Laboratory tests may become deranged with low platelets and abnormal clotting profiles. Plain abdominal radiography may reveal intra-mural gas, with intra-portal vein gas in severe cases. Free gas indicates intestinal perforation. Attempts have been made to develop profiles of inflammatory markers to aid in the diagnosis of NEC, but these have been largely frustrated (Edelson, 1999). Bell's classification (Bell et al, 1978) is now widely used to sub-divide groups of patients thought to have NEC into mild, moderate and severe forms of the disease (Bell Stages 1 [suspected], 2 [definite], 3 [severe]). With this clinical diagnostic process however it is inevitable that patients are misclassified both within NEC severity groups and between conditions yielding similar clinical signs to NEC. This makes the epidemiological study of NEC over time and the comparison of cases between different units difficult. The majority of infants who die in the acute phase of the disease succumb to MODS rather than bowel failure *per se*. Other systems affected by NEC include the liver (hyperglycaemia, hypoalbuminaemia, abnormal clotting, and hyperbilirubinaemia), the heart and cardiovascular system (low blood pressure, peripheral vasodilatation, cardiac dysrhythmias and gross oedema), the respiratory system (pulmonary oedema, poor oxygen exchange, and high ventilation pressures), the renal system (poor urine output, compromised concentration of urine, and acute tubular necrosis), and the central nervous system (prolonged hypo-perfusion with poor oxygen tension resulting in neurological impairment – which may be subtle or gross – on recovery from the acute phase of NEC)(Morecroft et al, 1994).

Treatment for NEC remains supportive (inotropes, artificial ventilation, blood products and haemofiltration) with an escalation of intestinal intervention from withholding feed to intravenous antibiotics and fluids to total parenteral

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nutrition. Necrotic or perforated bowel requires resection and either a primary anastomosis if the remaining intestine is clearly viable \pm a covering stoma, or exteriorisation of the ends with re-establishment of continuity at a later date.

Recurrent NEC has been described, emphasising the need for continued monitoring of the clinical situation after initial improvements have been achieved. Later sequelae of NEC include strictures within the surviving gut (occurring in 10% of survivors within a few weeks of an attack of NEC, and more frequently in defunctioned bowel), short-bowel syndrome and failure of the small bowel (inability of the remaining small bowel to provide enough absorptive surface area to sustain nutrition) leading to long-term total parenteral nutrition dependency. Small bowel lengthening and small bowel transplantation are the only therapeutic strategies available to address the significant loss of small bowel so early in life. Small bowel transplantation is still a rare clinical procedure with limited success, but small bowel lengthening procedures are becoming more widespread.

Indirect causes of intestinal ischaemia-reperfusion injury

Sepsis-induced critically low-flow

Sepsis is a carefully defined state in which the systemic inflammatory response syndrome (SIRS) is due to an infective agent (bacterial, viral, fungal, etc) (Bone et al, 1992). Sepsis and severe sepsis affected 9% and 6.3% of adults admitted to intensive care units in France (Brun et al, 1995). Severe sepsis and septic shock have a high mortality rate in adults despite advances in intensive care: 22 - 56% for severe sepsis (Anonymous, 1987; Ziegler et al, 1991; Brun et al, 1995) and 57% for septic shock (Ziegler et al, 1991). In children, severe sepsis affected 1 - 8 per 1000 live births in the USA in 1981 (Baley and Goldfarb, 1993). Mortality was between 20 and 30% and was inversely related to birth weight and female sex. In the 1990s the paediatric mortality rate from severe sepsis remained high at 10 - 34% (Duke et al, 1997) despite advances in the care of the critically sick.

Sepsis is associated with an initial hyperdynamic phase followed by a hypodynamic phase. The hyperdynamic phase is characterised by peripheral vasodilatation, widened pulse pressure, high blood pressure, tachycardia, fever and raised cardiac output (Kempley and Murdoch, 2000). The hypodynamic phase is characterised by peripheral vasoconstriction, narrowed pulse pressure, low blood pressure, hypothermia and reduced cardiac output. Cardiovascular sequelae of sepsis include mesenteric hypoperfusion, and sepsis is often accompanied by hypovolaemia or functional hypovolaemia as peripheral vasodilatation enlarges the intra-vascular space. This leads to sepsis-induced low-flow in the mesenteric circulation that may become critically low, and lead to mesenteric ischaemia (Hotchkiss and Karl, 1992). Many vasoactive molecules are up- or down-regulated during sepsis. For example, adrenomedullin is a potent vasodilator and is strongly up-regulated in early sepsis. It is possible that the shift from hyper- to hypo-dynamic phases of sepsis is related to a reduction in adrenomedullin or adrenomedullin availability (Yang et al, 2002). Abdominal signs indicating IIR injury during sepsis include non-specific, generalised abdominal distension, vomiting, diarrhoea, ileus and bloody stools (Muller W. and Smith L.L., 1963).

It is not clear why infection causes sepsis in some individuals. Sepsis is more likely with infection due to gram-negative organisms; some of the systemic manifestations of sepsis are mimicked by intravenous lipopolysaccharide (LPS) [found in the cell wall of gram-negative organisms](Muller W. and Smith L.L., 1963). A complication of assessing IIR injury during sepsis is the potential release of LPS from the intestines after IIR – this could propagate the SIRS initiated by the original septic insult and lead to a positive feed-back situation (more LPS released, worsening SIRS, more IIR injury, more LPS released from the gut). However, evidence for the release of LPS from the gut after IIR is lacking in man, and not universally found in animal models of IIR. Therefore this positive feedback loop remains an unproven hypothesis at present. Sepsis is treated with antibiotics to

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eliminate the infective insult, and supportive measures to combat systemic sequelae (inotropes, ventilation, renal replacement, etc).

Burns

Severe burns are a potent trigger of the SIRS and cardiovascular instability with an initial hyperdynamic phase followed by a hypodynamic phase. Splanchnic perfusion is compromised after significant burns (Herndon and Zeigler, 1993; Tadros et al, 2000). Post-burn splanchnic hypoperfusion may theoretically lead to release of endotoxin or intestinal bacteria into the bloodstream leading to post-burn sepsis, and the presence of sepsis worsens intestinal permeability (Ziegler et al, 1988; Tadros et al, 2003; Baron et al, 1994; Fleming et al). If burns are complicated by super-added infection, IIR injury becomes even more likely (Iglesias et al, 1998b). Burn injury leads to changes in intestinal morphology consistent with hypoperfusion (Spies et al, 2002).

The aetiology of burn-related IIR is partially elucidated by studies using cytokines or cytokine inhibitors: pre-treatment with interleukin-1-alpha before burn in pigs preserves mesenteric blood flow, increases oxygen delivery and consumption and reduces intestinal permeability increases and bacterial translocation (Tadros et al, 2003). Inhibiting TNF- α locally prevents burn-induced damage to intestinal mucosa in mice without sacrificing local mucosal cell proliferation (Spies et al, 2002).

Burns are treated by local restoration of skin coverage and supportive measures to abrogate the effects of the severe inflammatory insult induced.

Haemorrhage and cardiovascular insufficiency

Trauma remains the most common cause of death in children and young adults, and many trauma-related fatalities are due to uncontrolled haemorrhage. If the patient survives the initial haemorrhagic insult, many go on to suffer MODS (Bone et al, 1992) during recovery on the intensive care unit. During the initial haemorrhagic insult it is likely that an element of intestinal hypoperfusion is

present as vital organs are preferentially perfused (Scholander, 1963). This could lead to portal venous flow reduction, liver damage and an increase in intestinal permeability (Rush et al, 1988; Glasgow et al, 2000; Barr et al, 2000; Mongan et al, 2002). This latter factor may potentially facilitate the release of LPS into the portal circulation along with bacterial translocation, although it is noted that labelled intraluminal LPS did not cross the intestinal mucosa after haemorrhagic shock and resuscitation in rats despite transmucosal passage of whole *E. Coli* (Benoit et al, 1998). Benoit et al speculate that transmucosal 'passage' of LPS *in vivo* may be due to the release of bacterial cell wall fragments from killed translocated bacteria.

Global low-flow states may also be caused by cardiovascular insufficiency after myocardial infarction, myocardial stunning, cardiac surgery or pulmonary embolus. Intestinal ischaemia is a well-recognised complication of cardiac surgery – this may be related to induction of SIRS by cardio-pulmonary bypass as well as splanchnic hypoperfusion.

Treatment of global low-flow due to haemorrhage or cardiovascular insufficiency is aimed at restoring circulating volume and cardiac function.

Surgery

Intestinal and retroperitoneal surgery will inevitably cause a degree of IIR injury during intraoperative organ manipulation (Anup et al, 1999). Thoraco-abdominal and intra-abdominal vascular surgery is associated with more severe IIR injury that is likely to be exacerbated by pre-existing vascular disease within the abdomen. Ischaemic colitis after abdominal aortic aneurysm (AAA) repair is an uncommon [2.8% - 5% (Farkas et al, 1992; Jonung et al, 1991)] but potentially fatal complication [31% - >50% post-operative mortality (Farkas et al, 1992; Jonung et al, 1991)] of loss of the inferior mesenteric artery when a graft replaces the lower aorta. Post-operative colonic ischaemia after AAA repair (Delaney et al, 1999; Syk et al, 1998) is less likely after endovascular repair compared to open repair (Elmarasy et al, 2000). Endotoxaemia occurs after both thoraco-abdominal aortic aneurysm and AAA repair (Hafez et al, 2000; Roumen et al, 1993a) and is

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associated with increased intestinal permeability (Roumen et al, 1993b). Lower limb ischaemia-reperfusion injury has been shown to have adverse effects on the structure and function of the intestines (Yassin et al, 1997) and raises serum endotoxin and interleukin 6 levels (Yassin et al, 1996).

Treatment of surgery-related IIR is supportive initially, with escalating intervention dictated by clinical findings. Necrotic bowel must be removed, and post-ischaemic strictures of the colon after AAA-repair have been described.

Abdominal hypertension

Raised intra-abdominal pressure will compromise intestinal lymphatic and venous drainage if pressures approach capillary hydrostatic pressure. Abdominal hypertension has recently been recognised as a cause of intra-abdominal organ ischaemia on intensive care units (Ertel and Trentz, 2001). Abdominal hypertension is believed to be due to intra-abdominal oedema due either to the original insult (surgery, trauma) or to subsequent infection or inflammation. Some units now monitor intra-abdominal pressure routinely in patients at high risk of abdominal hypertension (via bladder pressure readings) so that if unphysiologically high pressures (greater than 20 - 30 mmHg) are recorded action may be taken (Williams and Simms, 1997). Clinical signs supporting the presence of raised intra-abdominal pressure are reduced compliance and distension of the abdomen, raised ventilation pressures and reduced urine output. Decompression of the abdomen is performed surgically and the abdomen is left open (laparostomy) with or without the use of a plastic membrane covering the intestines (a 'Bogota bag') to prevent excessive insensible loss of fluids during the period of laparostomy. When organ function has recovered and organ oedema has resolved, the abdomen can be closed again.

Portal hypertension

Raised portal vein pressure (greater than 10 mmHg) reduces portal venous flow through the liver, increases back-pressure in the portal vein and restricts

venous efflux from the intestines. Intestinal perfusion is compromised, intestinal ischaemia induced and intestinal barrier failure may occur.

Causes of portal hypertension are pre-, post- or intra-hepatic. Pre-hepatic causes include portal or splenic vein thrombosis, increased splenic venous flow (increased flow in the splenic vein preventing normal emptying of the portal vein) or increased portal flow (usually due to an arteriovenous fistula). Liver disease likely to cause this problem must involve a sufficiently large volume of the liver to prevent the compensatory dilatation of hepatic venules unaffected by the disease process relieving the back-pressure in the portal vein. Intra-hepatic diseases resulting in portal hypertension are pre-, intra- or post-sinusoidal. Pre-sinusoidal liver disease obstructs portal venules – the leading cause of this is schistosomiasis (the ova colonize and obstruct portal venules). Sinusoidal diseases leading to portal hypertension include alcoholic cirrhosis of the liver – collagen accumulates in the space of Disse restricting the flow of blood through hepatic sinusoids. In early cirrhosis fat deposition in hepatocytes restricts the circulation of fluid further. In later cirrhosis, post-sinusoidal portal hypertension may also be caused by regenerating nodules compressing hepatic venules. Other causes of cirrhosis include post-hepatic, auto-immune, drug-induced and congenital (for example haemochromatosis, Wilson's disease and α_1 -antitrypsin deficiency). Post-sinusoidal or post-hepatic disease causes hepatic venous hypertension and slows blood drainage from the liver. This may be caused by thrombus within the hepatic veins (Budd-Chiari syndrome) or right heart failure.

Treatment of portal hypertension is aimed at alleviating the cause. If treatment of the condition giving rise to portal hypertension (for example anti-coagulation for portal vein thrombosis) is not effective in relieving the high intra-portal pressure, pharmacological, surgical or radiological intervention is indicated. Some reduction in portal pressure may be seen with propranolol (isosorbide mononitrate may be added if insufficient relief is obtained with propranolol alone). Surgical treatment is definitive if the liver is replaced with a healthy transplanted

liver. Radiological positioning of a transjugular intrahepatic portosystemic shunt (TIPS) may provide temporary relief as a bridge to liver transplantation, if the liver is severely diseased. Surgically fashioned portosystemic shunts bypass the liver and allow the portal vein to empty into the inferior vena cava. This approach markedly reduces hepatic portal perfusion and is associated with encephalopathy. Hepatic encephalopathy in this situation may be due to a lack of detoxification of amino acids arising from the gut by the bypassed liver, and could be complicated by a greater than normal gut-derived LPS load from ischaemic intestine. Partial shunts, decompressing only the oesophagogastric region, are associated with less early encephalopathy and preserve hepatic portal perfusion (Benjamin and Papagrigoriadis, 1998).

Consequences of intestinal ischaemia-reperfusion injury

Intestinal ischaemia: local effects

Low oxygen tension results in a supply of oxygen inadequate to support aerobic respiration and has metabolic consequences for all cell types. A generalised oxygen sensing pathway is triggered by hypoxia – a process that involves phosphorylation (Bertges et al, 2002). Enterocytes can survive a brief period of hypoxia by switching energy production from aerobic to anaerobic respiration. Other cells present in the intestines are also affected by low oxygen tension. In rats leukocyte/endothelial cell interactions increase without the interruption of blood flow if oxygen tensions are low (Wood et al, 1999) and intestinal release of 6-keto-prostaglandin $F_{1\alpha}$ is markedly increased (Myers and Hernandez, 1992). Prostaglandins are endogenous vasodilators and increased production in response to hypoxia may assist in maximising mesenteric blood flow to increase local oxygen delivery.

The switch to anaerobic metabolism occurs at the expense of efficiency - only 2 molecules of adenosine triphosphate (ATP) are yielded per molecule of glucose if glucose is used anaerobically, compared to 38 if glucose undergoes

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oxidative phosphorylation. This can temporarily sustain ATP production for vital cellular functions such as ion-exchange pumps, but will eventually result in intestinal ATP depletion (Kuwabara et al, 2000; Vejchapipat et al, 2000) and the local accumulation of lactate (Vejchapipat et al, 2000). Lactate dissociates to form lactate anions and protons at intra-cellular pH, reducing local pH.

The capacity to sustain anaerobic respiration over the long term is limited by the consumption of fuel and the generation of toxic by-products (lactate and excess protons). Carbon dioxide accumulation and reduced glucose levels contribute to the toxic cellular environment by suppressing intra-cellular pH further and restricting the anaerobic fuel supply. Intercellular signalling molecules (for example cytokines, chemokines and growth factors) produced in response to a reduced oxygen tension persist in this stagnant local milieu for far longer than intended, resulting in over-stimulation of enterocytes and neighbouring cells. A further danger of this accumulation of signalling molecules is the restoration of blood flow to the gut: a surge of signalling molecules may enter the portal circulation, thus delivering the accumulated bioactive molecules to the next capillary bed encountered – the liver.

If intestinal ischaemia is prolonged, cell death occurs. Death occurs via both apoptosis and necrosis in rats (Ikeda et al, 1998). Tissue integrity is eventually adversely influenced by prolonged ischaemia both at the functional and the morphological levels, and the degree of disruption at both levels is proportional to the length and severity of oxygen deprivation (Park et al, 1990; Parks and Granger, 1986; Granger et al, 1981).

Intestinal reperfusion: local effects

Oxygen reintroduction

In an apparent paradox, further metabolic problems are encountered if the delivery of oxygen is restored either by the restoration of arterial inflow to the intestines or extra-vascular delivery of oxygen to tissues. The restoration of

oxygen delivery affects cellular metabolism. This is due to the toxic effect of an excess of molecular oxygen within cells, amplified by the altered configuration of certain enzymes after a period of low oxygen tension. One of the key enzymes involved is xanthine dehydrogenase/oxidase – inter-convertible forms of the same enzyme. The intestinal villous is the richest bodily source of xanthine dehydrogenase/oxidase and a concentration gradient is seen, increasing towards the villous tip. This may make the villous tip even more vulnerable to ischaemic damage. Under stress-free conditions, only 19% of the enzyme is in the form of xanthine oxidase making the disposal of xanthine via the reaction catalysed by xanthine dehydrogenase more likely (Parks et al, 1988).

Xanthine accumulates as a by-product of continued ATP use and degradation within hypoxic tissue. Xanthine dehydrogenase catalyses the conversion of xanthine to uric acid using nicotinamide adenine dinucleotide (NADH) as an electron acceptor. A significant proportion of intestinal xanthine dehydrogenase is converted into xanthine oxidase when local oxygen tension has been low for a prolonged period (Parks et al, 1988; Hirata et al, 1996). The mechanism of the conversion of xanthine dehydrogenase to xanthine oxidase is not clear, but may be via calcium-dependent proteolysis via inhibition of the energy-dependent calcium pump secondary to ATP depletion, leading to an influx of calcium that activates calmodulin-regulated intracellular proteases. This proteolysis pathway is also activated by TNF- α , interleukins-1 and -3 (IL-1, IL-3), neutrophil-derived elastase and activated complement fragment, C_{5a}. Alternatively, oxidation of sulphydryl groups to disulphides may effect the conversion (Schoenberg and Beger, 1993).

Xanthine oxidase also catalyses the conversion of xanthine to uric acid, but uses molecular oxygen as an electron acceptor. If molecular oxygen is newly supplied when xanthine oxidase is the predominant enzyme, the superoxide anion (O₂⁻) is generated by the transfer of an electron via two metallic intermediaries (Mo^{IV/VI} and Fe^{II/III}). The superoxide anion is one of a family of highly toxic

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reactive oxygen species (ROS) that behaves as a free radical. A free radical is an independent chemical species with at least one unpaired electron and is highly chemically reactive. Under normal circumstances the superoxide anion is a short-lived intermediary rapidly converted to hydrogen peroxide in a reaction catalysed by superoxide dismutase. This keeps the normal intracellular steady-state concentration of superoxide anions low (less than 10^{-11} molar) but does generate hydrogen peroxide, itself a toxic by-product. Superoxide dismutase levels are increased in rat gut tissue after prolonged reperfusion (Fu et al, 1997). If molecular oxygen is supplied in the presence of an elevated level of xanthine oxidase and/or the normal disposal route is disrupted, superoxide anions may persist and cause local tissue damage either directly or by reacting with other ROS to form more toxic entities. Superoxide anion may also be produced by other flavoprotein oxidases (for example aldehyde oxidase), the auto-oxidation of reduced quinines, catecholamines and thiols and (in trace quantities) when oxygen combines with haemoglobin.

Other ROS produced during reperfusion are hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^\cdot). Hydrogen peroxide is produced transiently by amine oxidase in the metabolism of catecholamines, and by superoxide dismutase in the normal metabolism of the superoxide anion as detailed above. Under these circumstances hydrogen peroxide is removed in a reaction catalysed by catalase, generating water and molecular oxygen. Hydroxyl radicals are produced in the Haber-Weiss reaction (also known as the Fenton reaction) in which superoxide anions and hydrogen peroxide react in the presence of transition metals (usually iron or copper, in the form of chelates or haemoproteins) to form hydroxyl radical, hydroxyl anion (OH^-) and oxygen. Hydroxyl radicals are more reactive and therefore more toxic than either superoxide anions or hydrogen peroxide. It may be that the toxicity of the latter two ROS is due mainly to their conversion to the hydroxyl radical rather than by direct effects. ROS are generated within activated

neutrophils later in reperfusion. The enzyme involved in intra-neutrophil ROS production is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

Cell components are damaged by oxidant attack by ROS, but ROS may also contribute to intercellular signalling if present in low concentrations (Thannickal, 2003). Mitochondrial membrane potential (MMP) maintenance is crucial for the normal functioning of mitochondria, which are in turn critical to the normal functioning of cells. MMP is reduced by exposure to ROS (Levrault et al, 2003). Increased activity of nuclear factor κ B (NF κ B) is noted after exposure of cells to ROS. This is probably effected via increased activity of protein kinase C, increasing overall phosphorylation within the cell, including phosphorylation of inhibitory protein κ B α (I κ B α) which is normally associated with NF κ B, keeping NF κ B in an inactive configuration. NF κ B is an important regulator of the gene transcription of cytokines and adhesion molecules (Lentsch and Ward, 1999) – therefore activation of NF κ B will induce the expression of bioactive molecules capable of initiating the inflammatory cascade and propagating an inflammatory response. Increased activity of NF κ B and activator protein-1 (AP-1) after ROS exposure induces the expression of heat shock proteins, nitric oxide synthetase, TNF- α and Fas ligand, upregulates intercellular adhesion molecule-1 (ICAM-1) expression and primes the cell for apoptosis (Fan et al, 2002; Takano et al, 2002).

Cell metabolism is directly affected by ROS in other ways. Deoxyribonucleic acid (DNA) and intra-cellular proteins are damaged by peroxidation of bases disrupting transcription and function. Actin in the cytoskeleton of the cell is attacked by ROS and undergoes excessive polymerisation that reduces cell pliability. Phospholipids and poly-unsaturated fatty acids are damaged by peroxidation by ROS that has both physical and chemical sequelae. Changing the charges and physical structures of phospholipids forming the bipolar bi-layer is important to the structure of cell walls and intra-cellular compartments, and can disrupt the integrity of cells directly leading to cell death. One of the by-products of lipid peroxidation is malonyldialdehyde (MDA) which

is used as a surrogate marker of lipid peroxidation and measured using TBARS (thiobarbituric acid reactive substances). MDA is increased in rat gut tissue after prolonged reperfusion (Fu et al, 1997). Phospholipase A₂ is activated after IIR by a mechanism that is not yet clear (Gijon and Leslie, 1997). It is known that ROS promote increased activity of phospholipase A₂ - this may contribute to IIR-induced phospholipase A₂ activation. Activation of phospholipase A₂ mediates agonist-induced arachidonic acid release from phospholipids within membranes for eicosanoid production including prostaglandins, leukotrienes, thromboxane and lipoxins. Membrane phosphatidylcholine is split to produce lysophosphatidylcholine, the concentration of which rises in direct proportion to the increase in activity of phospholipase A₂ after IIR. 1-O-alkyl phosphatidyl choline is also produced which is a precursor of platelet-activating factor (PAF) – a potent pro-inflammatory chemokine. Eicosanoids produced after phospholipase A₂ activation attract and activate leukocytes, inflame endothelial cells and affect the local microcirculation. Leukocytes attracted into injured tissue and activated go on to generate more ROS via a 'respiratory burst' – this causes a positive feedback loop, attracting and activating more leukocytes, as well as liberating more ROS locally. Endothelial/leukocyte interactions are crucial to the migration of leukocytes into IIR-injured tissue. ROS act via endothelial cell membrane-bound xanthine oxidase and NADPH oxidase to promote the translocation of pre-formed P-selectin to the cell surface very soon after exposure to ROS, and to upregulate the protein transcription of P-selectin and ICAM-1. This results in increased cell surface expression of P-selectin and ICAM-1 after ROS exposure (Takano et al, 2002).

ROS activate complement and generate complement fragments C_{3a}, C_{4a} and C_{5a}. C_{5a} can propagate the inflammatory cascade and is a potent stimulator of mast cells, acting on specific receptors to trigger mast cell degranulation. This releases pre-synthesised vasoactive amines (histamine and serotonin). These amines cause local vascular smooth muscle contraction leading to vasospasm, and increase vascular permeability locally. Prevention of complement activation after IIR injury

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to the small bowel reduces IIR-related intestinal damage: conversely, inhibition of C_{5a} inactivation worsens intestinal damage (Kimura et al, 1998).

Nitric oxide

Nitric oxide (NO) is produced primarily by the endothelium and is an important intermediate of the cell-signalling cascade involved in both maintenance of normal vascular tone and blood flow, and in oxidant tissue injury. NO is synthesised from L-arginine by nitric oxide synthetase (NOS), three isoforms of which have been identified. Constitutive NOS (cNOS) is calcium-dependent and is found in endothelial cells (eNOS – type 3) and neuronal cells (nNOS – type 1). Inducible NOS (iNOS – type 2) may also be expressed constitutively by some cells (and cNOS may under certain circumstances be induced) but is usually expressed in response to stimuli requiring greater NO production – for example reperfusion injury. Measuring NO directly is difficult: plasma nitrite levels are used experimentally as an indirect measure of NO (Doughty et al, 1998a).

NO has both direct and indirect effects (Anaya et al, 2002). Direct effects occur at low concentrations of NO (<1 µM) for brief periods and are governed by cNOS activity.

At low concentrations, NO protects tissues by regulating vascular tone, inhibiting platelet aggregation, smooth muscle proliferation and leukocyte adhesion, scavenging ROS, maintaining normal vascular permeability and immune defences and stimulating endothelial regeneration (Lefer and Lefer, 1999). Immediately on reperfusion there is a burst of ROS and a drop in cNOS leading to a drop in local NO in rats (Khanna et al, 2000). During ongoing reperfusion of formerly ischaemic tissue, it is thought that endothelial function becomes abnormal and less NO is released. A relative lack of NO results in a decrease in local vasodilatation that may contribute to 'no-reflow' (a phenomenon observed after IIR in which no blood will pass through apparently pristine blood vessels despite removal of all mechanical impediments to flow) and reduced inhibition of platelet aggregation and leukocyte/endothelial cell adhesion. The latter two

effects are exaggerated by the presence of ROS, especially hydrogen peroxide. These changes contribute to a positive feedback loop resulting in further reductions in flow, accumulation of leukocytes and increased platelet/leukocyte and leukocyte/endothelial cell interaction in the reperfused area. Constitutively expressed levels of NO are directly involved in the inhibition of NF κ B via I κ B α . NO prevents the phosphorylation of I κ B α and thus maintains the association between I κ B α and NF κ B. NO may also act to increase or maintain levels of I κ B α within the cell. If NO levels drop, overall levels of I κ B α drop and I κ B α is more likely to undergo phosphorylation, dissociate from NF κ B and allow NF κ B to become active. As previously discussed, NF κ B is a transcription factor involved in the regulation of gene expression. Therefore a drop in NO levels predisposes towards the release of inflammatory cytokines and paradoxically increases the likelihood that reactive nitrogen oxygen species are generated via the induction of expression of iNOS.

Excessive levels of NO can act directly to damage the actin cytoskeleton and relax inter-cellular tight junctions, increasing endothelial rigidity and endothelial permeability. DNA is attacked and de-aminated resulting in strand breaks, adenosine diphosphate (ADP)-ribosyl synthetase is activated, energy stores are depleted and cell death ensues.

Indirect effects of NO are mediated by reactive nitrogen oxygen species (RNOS). RNOS are produced at higher local concentrations of NO (> 1 μ M) that more likely to occur when iNOS has been expressed. They are formed by the combination of NO and oxygen to produce dinitrogen trioxide or, if NO scavenges the superoxide radical, to produce peroxynitrite. In the locally acidic conditions found in the intestines after IIR, NO is more likely to combine with oxygen to produce peroxynitrite. When peroxynitrite is applied to intestinal epithelial monolayers *in vitro*, enterocyte apoptosis is increased (Potoka et al, 2002; Sandoval et al, 1997) and cell markers reflecting local peroxynitrite production are also increased when cultured monolayers of intestinal cells suffer permeability increases after exposure to cytokines (Menconi et al, 1998; Chavez et al, 1999).

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These effects indicate a possible role for peroxynitrite in provoking increased intestinal permeability.

NO competes with oxygen within the mitochondrion at the level of cytochrome oxidase and when bound, NO reversibly inhibits the activity of cytochrome oxidase. As cytochrome oxidase is crucial to the generation of ATP via the respiratory chain, this mechanism provides an oxygen/NO dependent mechanism to regulate ATP production so that ATP is only produced when sufficient oxygen is available. If insufficient oxygen is available to displace NO, then cytochrome oxidase remains inhibited and ATP production is suppressed. However, if peroxynitrite (ONOO^-) is generated, it combines irreversibly with cytochrome oxidase and therefore prevents ATP production via the respiratory chain thus crippling oxidative phosphorylation even when sufficient oxygen is available for the efficient generation of ATP. Peroxynitrite also stimulates a specific calcium release pathway making cell apoptosis more likely after peroxynitrite generation (Potoka et al, 2002) as stated above. Prolonged reperfusion is associated with increased gut tissue (Fu et al, 1997) and plasma levels of NO (Tsuruma et al, 1999) in rats. These results may be commensurate with increased NO production due to iNOS induction after IIR.

Raising the level of endogenous NO available during IIR injury may help to preserve intestinal flow and prevent platelet aggregation on reperfusion in rats (Schleiffer and Raul, 1996). It can be seen that creation of reactive nitrogen oxygen species during reperfusion may make reperfusion injury worse, and the timing of NO supplementation is crucial. Supplementation of NO before IIR appears to be beneficial, but supplementation during reperfusion appears to make reperfusion injury worse - this may underlie the disappointing results of iNOS supplementation as a treatment for IIR injury - a case of too much NO too late.

Expression and release of bioactive molecules

Adhesion molecules

Interaction between leukocytes and endothelium is governed by adhesion molecules expressed on both the leukocyte and the endothelial cell (Godin et al, 1993). IIR induces the expression of P- and E-selectin and ICAMs-1, -2, and -3 by endothelial cells, and results in rolling and adhesion of leukocytes to endothelial cells – a precursor to migration of leukocytes into the interstitium. All these molecules are expressed in markedly increased numbers when the endothelium is inflamed or stimulated by inflammatory cytokines or bioactive molecules - for example LPS, IL-1, TNF- α , PAF and leukotriene B₄ (LTB₄). Leukocytes express L-selectin, which assists in slowing leukocytes to rolling speed within blood vessels and is shed after the leukocyte has been activated, and β_2 -integrins (including LFA-1 [lymphocyte function-associated antigen-1, also known as CD11a /CD18], Mac-1 [macrophage antigen-1, also known as CD11b/CD18] and p150-95 [also known as CD11c/CD18]), which permit firm adhesion of the leukocyte to the blood vessel wall via ICAMs. Leukocyte rolling is a crucial prelude to leukocyte stasis, firm adhesion and transmigration. Within the mesenteric microvasculature leukocyte rolling is governed by the presence of L-selectin on the leukocyte and P- and E-selectins on the endothelium. PAF has been shown to co-operate with P-selectin in promoting leukocyte/endothelial cell binding. P-selectin-induced leukocyte rolling increases PAF-receptor/PAF interaction that then triggers LFA-1 and Mac-1 expression by the tethered leukocyte. This allows the leukocyte to firmly adhere to the endothelium via ICAM-1 receptors (Duran et al, 1996; Scalia et al, 1999; Chen et al, 1997; Riaz et al, 2002). Leukocyte adhesion molecules are expressed constitutively, but remain inactive until exposed to chemokines, cytokines, antigens or mitogens. Leukocyte expression of CD11b/CD18 is increased after exposure to TNF- α in rats (Zeng et al, 2002). Activation occurs via a conformational change in the adhesion molecule that may be effected by phosphorylation. Local release of chemokines (interleukin-8 [IL-8], LTB₄, C_{5a} and

PAF) causes receptors on leukocytes to signal β_2 integrin activation via G proteins. B_2 integrins can then engage with receptors on the endothelium and firmly attach the leukocyte to the endothelium. Firm attachment is necessary to allow transmigration of leukocytes into the surrounding tissues. TNF- α induces firm attachment of leukocytes to endothelial cells at tight junctions, through which they migrate, and the tight junctions then reseal (Zeng et al, 2002).

A change-over period is found in the predominant type of adhesion molecule expressed by endothelial cells - this leads to flexibility in the immune response to inflammation. Immediately on reperfusion, P-selectin is expressed and PAF released. This results in the rapid recruitment of circulating polymorphonuclear leukocytes (PMNs, also known as neutrophils) to the reperfused area. If IL-8 (also known as cytokine-induced neutrophil chemoattractant - CINC) is also released early in reperfusion by cytokine-activated endothelium, these PMNs are more likely to transmigrate into the interstitium. PMNs exposed to CINC (IL-8) release proteases (Novak et al, 2003). Later in reperfusion, E-selectin is expressed and IL-8 released. This results in the predominant recruitment of other types of leukocyte (lymphocytes and monocytes) to the reperfused area. This switch in the type of leukocyte attracted by the reperfused area may be related to the 'friendly fire' damage caused by activated PMNs in the local tissues as they undergo respiratory burst (releasing ROS), degranulation (releasing proteases) and cause physical obstruction (after PMN death).

Leukocytes are pushed close to the endothelial lining of post-capillary venules by several factors pertinent to reperfusion after a period of ischaemia. Under resting conditions, the diameter of post-capillary venules is just larger than the diameter of circulating leukocytes. Red blood cells tend to occupy the central stream of flowing blood; therefore leukocytes are pushed against the lining of blood vessels at the periphery of the stream. After IIR, post-capillary venules dilate and blood flow slows, but this only increases red blood cell aggregation

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within the central stream of the blood vessel and pushes leukocytes against the blood vessel wall with greater force. Shear stress at the blood vessel wall is reduced by slower flow, and allows leukocyte rolling to occur. Circulating leukocytes can be attracted to areas of reperfused intestine via alterations in adhesion molecules in response to changes in local oxygen levels. Low oxygen tension followed by a restoration of oxygen levels causes a very rapid increase in the expression of P-selectin on mesenteric endothelial cells due to mobilisation of pre-formed P-selectin from Weibel-Palade bodies within endothelial cells (Ichikawa et al, 1997). Local attraction and endothelial adherence of leukocytes may lead to local capillary plugging and can worsen IIR by preventing local blood flow in rats (Beuk et al, 2000). Leukocyte interaction with blood vessel walls increased after IIR in the submucosa, but not in the muscular layers of the small bowel, indicating a higher relative reduction of flow within the submucosa after IIR. The number of firmly adherent leukocytes in post-capillary venules is also increased after IIR, potentially contributing to poor venous run-off in rats (Chen et al, 1997). Leukocyte migration after IIR is affected by raised blood alcohol levels, with amplification of neutrophil migration into the small bowel compared with IIR alone in rats (Tabata and Meyer, 1995).

Jejunal tissue is infiltrated by neutrophils and monocytes after IIR. Tissue levels of myeloperoxidase (MPO) and monocyte/macrophage markers clones 1 and 2 (ED-1 and ED-2) also rise, representing infiltration by and activation of neutrophils and monocytes (Chen et al, 1997; Hierholzer et al, 1999). After migration into tissues, circulating neutrophils undergo a 'respiratory burst' and generate ROS, MPO and elastase that are released into the local environment. Several investigators have shown that MPO activity in intestinal tissues is increased as neutrophils migrate into the interstitial space in the intestine after IIR (Yagihashi et al, 1998; Souza et al, 2000; Squadrito et al, 1997a; Squadrito et al, 1997b; Tsuruma et al, 1999; Altavilla et al, 1996; Sun et al, 2002; Sun et al, 1999; Wada et al, 2001; Hayward and Lefer, 1999; Park et al, 1990; Simpson et al, 1993;

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Kimura et al, 1998; Andoh et al, 1999; Oktar et al, 2002) but not after intestinal ischaemia only or sham operation (Savas et al, 1997). Depletion or enhancement of circulating neutrophil numbers before IIR has profound effects on intestinal metabolism after IIR - intestinal ATP levels recover better on reperfusion after depletion of neutrophil numbers with cyclophosphamide prior to IIR. It must be noted that platelet and lymphocyte levels are also suppressed by cyclophosphamide. Conversely, enhancing neutrophil numbers using granulocyte colony stimulating factor (G-CSF) results in impaired recovery of intestinal ATP after IIR. Neither of these neutrophil number manipulations change pre-IIR intestinal ATP levels in rats (Kuwabara et al, 2000). Jejunal levels of G-CSF messenger ribonucleic acid (mRNA) increase in the mucosa and in the muscularis after IIR in rats (Hierholzer et al, 1999), promoting PMN production. Functional activation of circulating neutrophils with AAA surgery has been shown to correlate with development of complications (Spark and Scott, 2001).

PMN activation after IIR is not inevitable - MPO did not rise in intestinal tissue after venous ischaemia only followed by reperfusion in rats (Park et al, 1990). A rise in intestinal MPO after was not recorded in weanling rats (6 weeks old) despite a measurable increase in intestinal permeability to ⁵¹Cr-labelled EDTA (ethylenediamine tetra-acetic acid). This may represent a threshold IIR injury below which neutrophil migration and activation is not triggered (Langer et al, 1995). Leukocyte adhesion and migration is normally but not necessarily associated with an increase in vascular permeability. Leukocytes that have successfully migrated into the tissues appear to require activation to induce vascular permeability changes.

Chemokines

Chemokines regulate the local inflammatory response by controlling leukocyte traffic and differentiation (Olson and Ley, 2002). Chemokines include IL-8 (or CINC), monocyte chemoattractant protein-1 (MCP-1), regulated on activation normal T cell expressed and secreted molecule (RANTES) and

macrophage inflammatory protein (MIP). Leukocytes are attracted to the highest local concentration of chemokines, which must bind to endothelial cell surfaces to elicit chemotaxis *in vivo*. Bound chemokines can cross-link to increase their effective local concentration. Chemokines interact with chemokine receptors on leukocyte surfaces and trigger cellular shape change (lamellapodia extension via cytoskeletal restructuring) and release of bioactive molecules (ROS from neutrophils, histamine from basophils and cytotoxic proteins from eosinophils). IL-8 and MCP-1 can trigger β_2 -integrin dependent firm adhesion of rolling leukocytes to endothelial cells expressing ICAM-1.

G-protein activation via chemokine binding activates a cascade of intracellular enzymes including phosphatidylinositol-specific phospholipase C leading to transient increases in cytosolic calcium concentration (from intracellular calcium stores) and protein kinase C activation. Limitation of chemokine activity can be achieved at the level of the activated chemokine receptor via phosphorylation by a G-protein receptor coupled kinase. The receptor is internalized and is no longer available for stimulation by chemokines. This limits the duration of leukocyte trafficking. Chemokine receptors may also be desensitized by serine phosphorylation of the receptor by a kinase activated by a different receptor. This pathway is protein kinase C dependent. Chemokine receptor expression is regulated in part by inflammatory stimuli. Maintenance levels of receptor expression are controlled by local levels of interleukins -2 and -4 (IL-2 and IL-4). Chemokine receptor expression is suppressed by interleukin-10 (IL-10) and increased by LPS, IL-1 β , TNF- α and interferon- γ (IFN γ) (Olson and Ley, 2002).

It appears that chemokines have a role in IIR-induced injury. IL-8 (CINC) rises in plasma and tissue with reperfusion in rats (Yagihashi et al, 1998; Tsuruma et al, 1999). Blocking IL-8 reduces I/R induced neutrophil accumulation in the lung in rats (Bless et al, 1999). MCP-1 levels are increased in the presence of ROS and may cause local tissue injury via activation of ICAM-1 on hepatic endothelial

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cells. If MCP-1 is neutralized or blocked, tissue damage in the liver after I/R is reduced (Olson and Ley, 2002).

Cytokines

Cytokines are soluble, low molecular weight glycoproteins serving to regulate the innate and specific immune systems. At low concentrations cytokines influence local cells (paracrine action) and can auto-stimulate the cell from which they are released (autocrine action). In higher concentrations, cytokines can influence distant organs and behave in an endocrine manner. Cytokines act on specific receptors on cell surfaces and occasionally in solution in plasma. Traditionally cytokines have been considered to be pro- or anti- inflammatory based on the overall action of the cytokine to induce inflammation (pro-) or promote healing (anti-). This division is now seen to be over-simplistic and it is clear that several cytokines could be considered to be both pro- and anti-inflammatory depending on the precise local and whole body milieu.

Traditional anti-inflammatory cytokines include interleukins 10, 13, 4 and 12 (IL-10, IL-13, IL-4 and IL-12) and IL-1 receptor antagonist (IL-1ra) – a soluble product secreted by stimulated monocytes. IL-1ra acts as an anti-inflammatory cytokine by competitively inhibiting the actions of pro-inflammatory interleukins 1 α and 1 β (IL-1 α and IL-1 β) at the IL-1 receptor. A hierarchy of anti-inflammatory action has been suggested by Ward (Ward and Lentsch, 1999) with IL-13 and IL-10 being the most anti-inflammatory. IL-13 is thought to reduce local pro-inflammatory signalling by preventing the breakdown of I κ B α within activated cells. This in turn prevents the activation and translocation of NF κ B to the nucleus and thus stops the up-regulation and transcription of ICAM-1, IL-1 β and TNF- α . The mechanism by which IL-13 prevents breakdown of I κ B α within the cell is not clear and may involve the inhibition of phosphorylation and ubiquitination of I κ B α , or use an anti-proteasome mechanism.

Traditional pro-inflammatory cytokines include TNF- α , interleukins 1 α , 1 β , 2, 6, 15 (IL-1 α , IL-1 β , IL-2, IL-6, IL-15) and IFN- γ . Some pro-inflammatory

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cytokines are released early after an inflammatory stimulus – for example TNF- α and IL-1 β ; pro-inflammatory cytokines released as part of the later inflammatory cascade include IL-1 α , IL-2, and IL-6. The change in predominant pro-inflammatory cytokine is hypothesized to help to ‘fine-tune’ the inflammatory response to ongoing inflammation. Early cytokines stimulate PMNs that circulate and respond to early inflammation. Later cytokines attract and stimulate a different profile of leukocytes – monocytes, basophils and lymphocytes – that are more important in a sustained inflammatory response.

After IIR injury, both pro- and anti-inflammatory cytokines have been measured in portal and systemic blood and in tissue samples. Measurements include the active cytokine, cytokine converting enzymes and cytokine mRNA in intestinal and remote organ tissue. This approach can inform us about the relative levels of different cytokines during intestinal ischaemia and reperfusion, and may assist in understanding the relative roles of each cytokine and the interactions between different cytokines during IIR. Genetic knockout animals lacking in a single cytokine or monoclonal antibodies against a single cytokine may inform us of the role of that cytokine, but this reductionist approach may fail to take into account the redundancy of the inflammatory response.

Of the cytokines that have been investigated after IIR, I will consider TNF- α , IL-1 β , IL-6 and IL-10.

TNF- α is produced largely by mononuclear cells in response to signal transduction after an inflammatory stimulus. Expression is controlled by intracellular transcription factors such as NF- κ B. TNF- α is pyrogenic, activates the clotting cascade, induces the expression of adhesion molecules by leukocytes and endothelial cells, potentiates the synthesis of IL-1 β and stimulates the release of IL-6, IL-8 and IL-10. TNF- α acts on cell via TNF-receptors 1 and 2 (p55 and p75). The cytosolic domains of these receptors recruit different proteins that transduce the TNF- α signal further. Both promote the translocation of NF- κ B into the nucleus. TNF-receptor 1 can also recruit tumour necrosis factor-alpha receptor [TNFR]-

associated death domain proteins (TRADDs), the over-expression of which also results in cell death and the activation of caspase-1 (also known as IL-1 β converting enzyme). TNF- α can induce cell death more directly by binding to TNF-receptor 1 whose death domain can initiate cell apoptosis without further intermediates. Both TNF-receptor 1 and 2 exist in soluble forms and can act as TNF- α antagonists by binding TNF- α and preventing cell-bound receptor binding. They may also act as agonists by increasing the half-life of TNF- α as a bound molecule which can then dissociate from the soluble receptor and bind to a cell-bound receptor and stimulate the cell (Dinarello, 2000). Several investigators have demonstrated rises in serum or plasma TNF- α after IIR in both systemic and portal circulations (Squadrito et al, 1997a; Fu et al, 1997; Lane et al, 1997; Tsuruma et al, 1999; Yamagishi et al, 2002; Yao et al, 1996). Intestinal tissue levels of TNF- α have also been demonstrated to rise after IIR indicating local production of TNF- α (Wada et al, 2001; Souza et al, 2000), one source of which is the ileal mucosa (Grotz et al, 1999). There is great variability in the levels of TNF- α measured after IIR. This may be related to the severity of insult, the timing of sampling and possibly the species of animal model used (Yamamoto et al, 2001a; Squadrito et al, 1997b; Altavilla et al, 1996; Dowdall et al, 2002; Bitterman et al, 1991; Yagihashi et al, 1998; Yao et al, 1997; Bathe et al, 1998a; Yao et al, 1995; Horie et al, 2002). In experiments using graded hypoxia as a surrogate of IIR, TNF- α production does not increase with 'reperfusion' unless endotoxin is also present during 'ischaemia' (Ding et al, 2001; Stallion et al, 2002). This demonstrates the inter-dependence of TNF- α and endotoxin levels in IIR injury. A positive correlation between peak systemic endotoxin and TNF- α levels has been demonstrated after IIR (Yao et al, 1997), although it is interesting to note that the peak in endotoxin levels occurred after 30 minutes of reperfusion and that in TNF- α after 2 hours of reperfusion.

IL-1 β is an early pro-inflammatory cytokine released by monocytes soon after stimulation by an inflammatory stimulus. In common with TNF- α , IL-1 β is pyrogenic, can activate the clotting cascade, induces the expression of adhesion

molecules and stimulates the synthesis of IL-6, IL-8 and IL-10. IL-1 β potentiates the synthesis of TNF- α , but unlike TNF- α cannot directly initiate cell death. Control of IL-1 β expression is by NF κ B and other transcription factors. IL-1 β acts on cells via membrane-bound receptors; this leads to a series of intra-cellular events including multiple protein phosphorylations (for example phospholipase A₂ activation leading to a rapid release of arachidonic acid) and activation of phosphatases. IL-1 β binding induces transcription factors NF κ B and AP-1. IL-1 β acts synergistically with TNF- α to induce NO and IL-8 production; in this context, NO mediates cell death (Dinarello, 2000). Investigators have shown that IL-1 β levels rise in systemic serum and intestinal tissue after IIR (Sun et al, 1999; Sun et al, 2002; Yamamoto et al, 2001a; Takeyoshi et al, 1999; Farber et al, 1999) but results are not consistent across studies: paradoxically, other investigators have shown no increase in serum IL-1 levels after IIR (Bitterman et al, 1991; Gurley et al, 1997). These studies highlight the difficulties in comparison of results between different studies using different species, with tissue and blood samples obtained at different stages of IIR injury.

IL-6 is a potent inducer of the acute-phase protein response in hepatocytes and enterocytes, and IL-6 release can be induced by leukocyte exposure to LPS and IL-1 β , and after induction of the heat shock response – these facts mean that IL-6 is usually classified as a pro-inflammatory cytokine. However, IL-6 has potent anti-inflammatory actions as well. After binding to a specific α receptor, IL-6 down-regulates the production of IL-1 β and TNF- α and inhibits the production of other pro-inflammatory cytokines and chemokines (IFN- γ and MIP-2), without reducing the production of IL-10 and transforming growth factor- β (anti-inflammatory cytokines). IL-6 induces the synthesis of glucocorticoids and promotes the production and release of IL-1ra and soluble TNF-receptors (Opal and DePalo, 2000). IL-6 levels in systemic serum rise with IIR injury (Sun et al, 1999; Yao et al, 1997; Lane et al, 1997). Clinical studies have demonstrated a rise in systemic IL-6 levels after AAA repair and intussusception – both associated with IIR injury (Syk

et al, 1998; Willetts et al, 2001). Quantification of IL-6 levels after IIR shows the same variability noted in measurements of TNF- α and IL- β (Bitterman et al, 1991; Dowdall et al, 2002). The origin of IL-6 after IIR has been examined: IL-6 levels rise with reperfusion only and levels of IL-6 in the portal and hepatic veins are consistently higher than in the carotid artery indicating a splanchnic origin (Bathe et al, 1998a). Jejunal IL-6 mRNA is expressed at higher levels in the mucosa and in the muscularis after IIR, supporting the concept of the intestine as an important source of IL-6 after IIR (Hierholzer et al, 1999). Hypoxia (mimicking ischaemia) suppresses ileal membrane production of IL-6 and decreases ileal membrane electrical resistance; restoration of normal oxygen levels (mimicking reperfusion) increases IL-6 production by mucosal cells and normalises ileal electrical resistance (Ding et al, 2001). The inter-dependence of cytokine release in response to IIR has been demonstrated by IL-10 deficient mice who release more IL-6 into the systemic circulation than normal mice after identical IIR insults (Stallion et al, 2002).

IL-10 is one of the most important anti-inflammatory cytokine discovered to date. IL-10 is predominantly synthesised by CD4 positive T₂-helper cells, monocytes and B lymphocytes. It has a specific cell-membrane receptor to which it binds with high affinity. On binding, monocyte production of TNF- α , IL-1 β , IL-6, IL-8, IL-12, G-CSF and MIP-1 α and -2 α are inhibited. Cell-surface expression of major histocompatibility complex class II, B7 accessory molecules and CD14 (an LPS recognition and signalling molecule) are all inhibited. Neutrophil and natural killer cell cytokine production is blocked. NF κ B translocation to the nucleus after LPS stimulation is prevented, and mRNA of pro-inflammatory cytokines is degraded. TNF- α receptor expression is reduced and TNF-receptors are shed into the circulation. IL-10 rises after IIR injury in both experimental and clinical settings (Tsuruma et al, 1999; Doughty et al, 1998b; Edelson et al, 1999). IL-10 levels have been supplemented pre- and post- reperfusion in animal models of IIR. Systemic serum levels of IL-6 and TNF- α were reduced regardless of when IL-10 levels were supplemented (Lane et al, 1997). Remote lung injury and local

histological damage were also reduced in IL-10 treated animals. Preliminary studies of recombinant IL-10 as a clinical treatment for inflammatory bowel disease show promise in Crohn's disease, but not in ulcerative colitis (Lane et al, 1997). Stallion et al (Stallion et al, 2002) used IL-10 knockout mice and found that IL-10 deficient mice were not more susceptible to IIR compared to wild-type litter mates, with identical mortality rates and histological damage to the intestines. IL-10 deficient mice did release more IL-6 into the systemic circulation than normal mice. TNF- α levels in both tissue and serum were below the limits of detection. Stallion et al explained these findings by the assumed redundancy within the cytokine response system that may compensate for the loss of a single cytokine. In contrast, Zingarelli et al (Zingarelli et al, 2001) found that IL-10 deficient mice had much higher mortality rates than wild-type mice. IL-10 deficient mice also had much greater intestinal histological damage and local neutrophil infiltration (as shown by higher tissue levels of MPO). Lipid peroxidation appeared to be more severe (higher levels of MDA) and nitrate oxygen free radicals generated more nitrotyrosine. In response to this IIR insult, IL-10 deficient mice produced higher plasma TNF- α and IL-6 levels and more endothelial expression of P-selectin and ICAM-1. Zingarelli et al hypothesised that endogenous IL-10 functions as a counter-inflammatory cytokine regulating the early stress related gene response governing adhesion molecule and inflammatory cytokine gene expression. This regulates leukocyte adhesion and excitation under stressful conditions. IL-10 does not affect (constitutive) ICAM-1 expression so is unlikely to be important in the stress-free state. This latest point may explain the difference in reported results between Stallion et al and Zingarelli et al, as Stallion et al's insult was milder. Stallion et al occluded only the SMA for between 20 and 50 minutes, and permitted 2 hours of reperfusion; Zingarelli et al occluded both the SMA and coeliac axis for 45 minutes, and permitted 6 hours of reperfusion. Thus the insult inflicted by Zingarelli et al would have caused ischaemia of a larger part of the gut (foregut and midgut) and reperfusion was permitted for three times as long as in Stallion et

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al's experiment. Stallion et al may therefore not have reached a hypothetical threshold of 'stress' above which IL-10 functions as a counter-inflammatory cytokine, and the synergy between TNF- α and endotoxin was excluded (TNF- α levels were undetectable in Stallion et al's experiment) reducing the inflammatory response to the IIR insult. A possible role for IL-10 is proposed in the protective effect of the heat shock response (HSR). Successful induction of the HSR is associated with low levels of TNF- α , IL-8 and high levels of IL-10 after IIR; if the HSR is blocked, IIR results in higher levels of TNF- α , IL-8 and lower levels of IL-10 (Tsuruma et al, 1999). The protective effect of the HSR in sepsis is associated with a rise in systemic serum IL-10 and is reversed by the administration of anti-IL-10 antibodies (Wang and Hasselgren, 2002), reinforcing the hypothesized role of IL-10 in HSR protection against the consequences of inflammation. Most work on the interaction of the HSR, IL-10 and I/R has been performed using cardiac material (Xi et al; Gowda et al, 1998) but lies outside the scope of this thesis.

Other bioactive molecules

Soluble complement receptor 1 administration or blocking complement activation ameliorates the effects of IIR on the intestine, providing indirect evidence of complement activation during IIR (Spain et al, 1999; Kimura et al, 1998; Zhao et al, 2002). Conversely, prevention of inactivation of C_{5a} worsens local IIR-related injury (Kimura et al, 1998).

Several investigators have found that IIR increases circulating levels of vasoactive neuropeptides - raised concentration of endothelins 1 and 2 in the portal circulation (Yegen et al, 1994; Oktar et al, 2002), neuropeptide Y, vasoactive intestinal peptide and endothelin-1 in the systemic circulation (Dovgan et al, 1996), met-enkephalin-like immunoreactivity and noradrenaline, but not adrenaline, and serotonin in both circulations (Aneman et al, 1996; Nakamura et al, 2001). The source of serotonin appears to be degranulation of mast cells in the mucosal membrane (Kimura et al, 1998; Andoh et al, 1999). Mucosal mast cells and serotonin appear to be involved in local tissue damage - rats genetically deficient

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in mucosal mast cells suffer only mild epithelial lifting at the villous tip after IIR (Andoh et al, 1999). Each vasoactive peptide potentiates the action of noradrenaline on the vasculature. If similar vasoactive peptides are released with IIR, the local vasculature will be bathed in a vasoconstrictive milieu.

Serum protease inhibitor levels are reduced in rats after IIR (Sun et al, 1999; Sun et al, 2002). This is associated with reductions in circulating levels of albumin, α_1 -macroglobulin, antithrombin III and α_2 -antiplasmin. Ileal tissue levels of prostaglandin E₂ and leukotriene C₄ levels rise after IIR (Yegen et al, 1994) but intestinal release of 6-keto-prostaglandin F_{1 α} is markedly reduced compared to intestinal ischaemia alone (Myers and Hernandez, 1992). Myers et al hypothesized that ROS generated on intestinal reperfusion inhibit local synthesis of prostaglandins. Prostaglandins normally help to control mesenteric blood flow by acting as endogenous vasodilators – inhibition would therefore result in relatively reduced mesenteric blood flow on reperfusion. This was confirmed in their experiment. In contrast, a large increase in intestinal eicosanoid release – 6-keto-prostaglandin F_{1 α} (a stable metabolite of prostaglandin I₂) after IIR has been described by Turnage (Turnage et al, 1995a).

The systemic recirculation of bioactive molecules released from the intestines after IIR may provide the so-called ‘second hit’ hypothesized to induce SIRS by some theorists after priming by the initial IIR insult (Grotz et al, 2001; Partrick et al, 1996).

Increased intestinal permeability after IIR: release of endotoxin and bacterial translocation.

The functional disruption of enterocytes affected by IIR injury can result in compromise to barrier function (Grotz et al, 1999; Sun et al, 2002; Schleiffer and Raul, 1996; Khanna et al, 2001; Dowdall et al, 2002; Yamagishi et al, 2002), with increased permeability to ⁵¹Cr-labelled EDTA (Langer et al, 1993b; Langer and Sohal, 1992), phenol red (Grotz et al, 1999) and fluorescein isothiocyanate [FITC]-labelled LPS (Drewe et al, 2001). Increased intestinal permeability after IIR is

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exacerbated by inhibition of cyclooxygenase (with indomethacin) or intravenous prostaglandin E₂ in rats (Langer et al, 1995).

Plasma-to-lumen clearance of ⁵¹Cr-labelled EDTA is also increased after IIR, indicating a greater mucosal permeability to blood-borne molecules after IIR (Udassin et al, 1998; Kimura et al, 1998). Plasma levels of intestinal fatty acid binding protein (a marker of intestinal damage) increase after IIR in rats (Beuk et al, 2000) indicating release of enterocyte contents into the circulation after IIR.

IIR leads to the release of endotoxin into the circulation (Yamamoto et al, 2001a; Yao et al, 1997; Turnage et al, 1994; Yao et al, 1995; Ohara et al, 2001; Horie et al, 2002; Yamagishi et al, 2002; Gaffin et al, 1986; Gathiram et al, 1989) but the levels achieved in the portal and systemic circulations vary with the severity of insult applied and the species of experimental animal used. In contrast, Bathe found that IIR resulted in no increase in serum endotoxin in pigs at any site sampled or excess of endotoxin in portal venous or hepatic venous blood compared to carotid arterial samples. Bathe et al hypothesised that this may be due to the release of endotoxin into peritoneal fluid or lymph (Bathe et al, 1998a).

There may be direct effects of local endotoxin release on the intestines and other organs: intravenous LPS increases jejunal and ileal NOS activity, affecting both constitutive and inducible NOS in rats (Cullen et al, 1999). Endotoxin introduced directly into the right atrium in dogs is associated with net release of lactate from the lung and reduced clearance of lactate by the liver, skeletal muscle, kidney and gut (Bellomo et al, 1996). Stimulating rat ileal membranes after IIR *in vitro* with endotoxin results in a marked increase in release of TNF- α and IL-6 release (Grotz et al, 1999).

In addition to the increase in intestinal permeability and endotoxin release after IIR, the intestines may become permeable to viable bacteria. Rat ileal mucosa is found to be permeable to bacteria after IIR, but mesenteric lymph nodes remain sterile with a mild insult (Grotz et al, 1999). Prolonged reperfusion after intestinal ischaemia results in positive bacterial culture with intestinal organisms in the liver,

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spleen and mesenteric lymph nodes (Fu et al, 1997; Yao et al, 1995; Ohara et al, 2001; Kucukaydin et al, 2000).

A possible mechanism for bacterial translocation after IIR is suggested by Clark et al, who found that exposure to TNF- α alone could not provoke bacterial translocation through a human colonic cancer cell-derived monolayer (with predominantly enterocyte characteristics) but TNF- α exposure combined with glutamine depletion did result in increased bacterial translocation. *Escherichia Coli* were transported through cells in vacuoles and did not appear to pass through the monolayer via the paracellular route (Clark et al, 2003). Intestinal glutamine rises with intestinal ischaemia and falls towards normal levels with intestinal reperfusion in rats, possibly indicating inhibition of glutamine oxidation in the ATP-poor intestinal environment after IIR (Vejchapipat et al, 2000), and a functional deficit in glutamine use.

Histological changes after IIR

IIR results in histological damage to the intestines that is proportional to the length of ischaemic time and period of reperfusion. Chiu et al (Chiu et al, 1970) used dogs to develop a descriptive scale (scores 0 – 5 inclusive) to describe the progressive intestinal injury seen after IIR. Many investigators have used this scale to quantify the severity of intestinal injury after IIR, and to compare intestinal injury across different treatment groups (Savas et al, 1997; Yagihashi et al, 1998; Lane et al, 1997; Ohara et al, 2001; Ward et al, 2000; Kazez et al, 2000; Dowdall et al, 2002). Similarly to the variation in cytokine levels reported after IIR, differences in mean histological score after IIR may reflect inter-species variations in response to IIR injury, as well as differences in insult type and duration. Some investigators have reported surprising little or no histological damage to the intestines after IIR (Ikeda et al, 1998; Kuenzler et al, 2002). There is a significant linear relationship between intestinal ischaemic time and histological injury score in mice (Farber et al, 1999). Mild histological damage increases early in intestinal reperfusion and decreases with prolonged intestinal reperfusion in rats (Hierholzer et al, 1999).

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Park (Park et al, 1990) used rats to develop an extended descriptive scale which is identical to the Chui scale for scores 0 – 5, but subdivides more severe damage into scores 6,7 and 8. Park demonstrated that the pattern of histological damage seen after IIR depended on the type of intestinal ischaemia. Brief purely venous ischaemia does not produce any histological damage. Longer purely venous ischaemia results in progressive loss of villous height, and damage is proportional to the duration of ischaemia. Significantly, no exacerbation of intestinal damage is seen if reperfusion is permitted. Purely arterial ischaemia results in the same degree of intestinal damage after ischaemia only that was noted after venous ischaemia. Reperfusion resulted in significantly more histological damage to the intestines than seen with arterial ischaemia only.

Computerised morphometric analysis has been used to quantify histological damage to the intestine with a series of measurable parameters (surface index [= mucosal surface length/serosal length of gut]; average villous height; average villous thickness) and has demonstrated reproducible intestinal injury after IIR in rats (Puglisi et al, 1996). In a further attempt at a quantitative analysis of intestinal tissue damage after IIR, histological grading of ischaemic rat intestine has been compared with reduction of nitro blue tetrazolium salts in mitochondria to blue formazon dye which can be measured spectrophotometrically (Powell et al, 1995). Reduction of nitro blue tetrazolium salts is impaired after 30 minutes of intestinal ischaemia and decreases in proportion to the duration of ischaemia up to 120 minutes of ischaemia [$r = -0.76$]. Comparison with histological score using Chui's 0 - 5 scale also revealed a negative correlation [$r = -0.77$]. Reperfusion of the intestine was not permitted. Using a threshold of $<30 \mu\text{g}/\text{mg}$ protein, nitro blue tetrazolium dye predicted intestinal ischaemia of greater than 30 minutes with a specificity of 100% and a sensitivity of 94% in this experiment.

Several investigators have produced descriptions of the features of histological damage to the intestines seen after IIR in rats: mucosal destruction, villous loss, epithelial cell loss, intramucosal haemorrhage and inflammatory cell

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infiltrate (Ward et al, 2000); villous necrosis, villous fibrosis, intestinal wall necrosis and perforation (Pitt et al, 1991); tissue haemorrhage (Souza et al, 2000); crypt destruction – an irreversible histological change (Hirata et al, 1996); disruption of the villous tip, loss of villous height, dilated capillaries, haemorrhage and ulceration (Kimura et al, 1998); loss of crypts and villi and an inflammatory cell infiltrate into the mucosa (Shah et al, 1997)

Intestinal metabolic derangement after IIR

Intramucosal pH (pHi) has been shown to fall after IIR in dogs (Takeyoshi et al, 1999), pigs (Bathe et al, 1998a), rats (Ohara et al, 2001; Kuwabara et al, 2000) and humans (Syk et al, 1998).

Intestinal tissue ATP (Kuwabara et al, 2000) and phosphocreatine fall rapidly to become undetectable after 20 minutes of intestinal ischaemia in rats. On reperfusion, rat intestinal ATP levels make only a partial recovery if ischaemic time is greater than 30 minutes (Blum et al, 1986). Relative changes in ATP levels (quantified by *in vivo* magnetic resonance spectroscopy) exhibit a linear correlation with tissue extract ATP levels measured by HPLC (high performance liquid chromatography). Intestinal inorganic phosphate (Pi) rises with intestinal ischaemia and recovers with reperfusion in proportion to the intestinal ischaemic insult in rats (Sato et al, 1996). Tissue ATP levels fall and tissue Pi levels rise within 3 minutes of intestinal ischaemia. This is more rapid than ultrastructural changes in enterocytes observed with electron microscopy, which are only apparent after 10 to 15 minutes of intestinal ischaemia in rats (Kass et al, 1987). Energy charge ($\frac{[ADP/2] + [ATP]}{[ADP] + [ATP] + [AMP]}$) rises with IIR in rats (Yamada et al, 1995) but ATP concentration is the best predictor of intestinal viability: energy charge does not reliably predict intestinal viability in rats (Sato et al, 1999). Intestinal mucosal cyclic guanosine monophosphate (GMP) content falls after IIR in rats (Schleiffer and Raul, 1996). Intestinal ATP content falls after IIR and a negative correlation exists between the intestinal content of ATP and the proportion of xanthine dehydrogenase/oxidase

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present in the oxidase form in rats (Hirata et al, 1996). A mechanism for the fall in ATP after IIR may be extrapolated from the work of Fellman, who hypothesized that it is the reperfusion of formerly ischaemic tissue that results in brain damage after perinatal asphyxia. Ischaemia reduces cell ATP levels allowing accumulation of calcium and sodium within the cell, causing primary energy failure, cell swelling and some cell death. Reperfusion partially restores cell ATP levels, but these are not sustained and secondary energy failure, possibly related to generation of ROS in reperfused tissue, leads to apoptotic cell death and delayed loss. Outcome is proportional to the degree of primary energy failure. The delay in apoptotic cell death after reperfusion may allow for intervention to prevent secondary energy failure and cell loss after reperfusion, and could potentially improve eventual outcome (Fellman and Raivio, 1997).

Intestinal reperfusion after an ischaemic insult results in partial recovery of glucose, succinate, lactate, alanine, glutamine and glutamate levels, but no recovery of phosphatidylcholine, glycerophosphocholine, choline, ATP, ADP or Pi in rats (Vejchapipat et al, 2000). Vejchapipat et al hypothesised that inhibition of oxidative phosphorylation results in accumulation of tricarboxylic acid cycle intermediates (succinate) and depletion of ATP stores. Anaerobic use of glucose to produce ATP generates excessive lactate that dissociates to yield lactate anions and protons. Hydrolysis of ATP yields ADP, Pi and protons. Increased proton release results in acidosis that inhibits the activity of pyruvate dehydrogenase, making more pyruvate available for conversion into lactate and alanine. These reactions increase lactate and alanine production and deplete pyruvate. Protein catabolism consumes amino acids, and glutamine oxidation is inhibited, as it is an energy-consuming process. Choline levels may rise because synthesis of phospholipids (an energy consuming process) is halted and/or choline is liberated when phospholipids are peroxidised. Pi may have been released from several sources, including phospholipid membranes after lipid peroxidation or from the breakdown of high-energy phosphates (ATP and ADP). Restoration of blood flow

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does not restore ATP levels implying that irreversible energy failure has occurred with this severe insult.

Further biochemical abnormalities of the intestines after IIR have been described by other groups. Intestinal lactate dehydrogenase levels fall after IIR in mice (Zhao et al, 2002) and rats (Wada et al, 2001). Ileal mucosal diamine oxidase activity is reduced after IIR in rats (Yao et al, 1995). Evidence of lipid peroxidation has been described by other investigators: ileal MDA tissue levels rise after IIR in rabbits (Aydemir et al, 1999) and rats (Yegen et al, 1994; Kazez et al, 2000), but not after intestinal ischaemia only or after sham operation in rats (Savas et al, 1997).

It is possible that the local biochemical effects of IIR are exaggerated by the experimental situation. Biochemical changes are found in the intestinal mucosa of rats after minimal surgical manipulation under general anaesthetic - mucosal alkaline phosphatase activity is reduced, α -tocopherol content is reduced, MDA content is increased, glucose transport is reduced in isolated mucosal membrane vesicles, and arachidonic acid is generated by activated phospholipase A₂. Phosphatidyl choline and phosphatidyl ethanolamine are reduced and lysophosphatidyl choline, lysophosphatidyl ethanolamine and linoleic acid are increased (Prabhu and Balasubramanian, 2002). Biochemical studies of the intestines after IIR must therefore be interpreted with caution, and only after comparison to appropriate controls.

Cell death and apoptosis after IIR

Cell death after intestinal ischaemia-reperfusion has previously been described as predominantly due to necrotic cell death. An increasing body of evidence is emerging that programmed cell death (apoptosis) has an important role in the loss of cells after IIR (Ikeda et al, 1998; Farber et al, 1999; Kuenzler et al, 2002; Shah et al, 1997). The mechanism behind the apoptotic loss of intestinal cells is not yet clear, but is thought to involve the following mechanism: TNF- α and IF- γ released after IIR inhibit complexes I and II of the respiratory chain. TNF- α and IL-1- β also trigger mitochondrial hypermetabolism, increasing respiratory quotient

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and oxidative phosphorylation rate and decreasing energy charge. Calcium turnover is increased as calcium is released from dysfunctional mitochondria and calcium uptake is increased from the surrounding extra-cellular fluid. Rising calcium concentration in the cytosol activates destructive enzymes (proteases, phospholipases and endonucleases) and is a potent trigger of apoptosis leading to the eventual loss of the whole cell.

The relative roles of ischaemia and reperfusion are partially elucidated by the work of Van den Hoek (Vanden Hoek et al, 2003), who found that reperfusion rather than simulated ischaemia lead to apoptotic cell death in embryonic chick cardiomyocytes. A simulated ischaemic insult followed reperfusion led to 50% cell death with substantial release of cytochrome C after 5 minutes of reperfusion, and caspase activation after 1, 2 and 3 hours of reperfusion. A purely ischaemic insult of equal total duration led to 10% cell death without cytochrome C release or caspase activation. Reperfusion of formerly ischaemic cardiomyocytes in the presence of a caspase inhibitor reduced the number of cells dying and improved functional recovery. Reperfusion in the presence of anti-oxidants reduced cytochrome C release, nuclear condensation and cell death. Van den Hoek et al speculated that ROS trigger changes in mitochondria resulting in the intracellular release of cytochrome C. This leads to the activation of caspase and to cell death by apoptosis, probably via α -fodrin cleavage after activation by caspase-3.

Blood flow after IIR

IIR disrupts the normal pattern of intestinal perfusion. In the experimental situation this can be induced and monitored easily, but in patients onset and recovery of intestinal ischaemia may be harder to predict. Intestinal reperfusion is a dynamic entity, and several groups have shown altered intestinal perfusion some time after an IIR insult.

A predictable and significant decrease in intestinal mucosal blood flow during intestinal ischaemia has been demonstrated by several groups. It is of interest that mucosal blood flow is rarely restored to pre-ischaemic levels on

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intestinal reperfusion in rats (Schmeling et al, 1989; Turnage et al, 1991; Ohara et al, 2001). Hyper- followed by hypo-dynamic blood flow is a recognised pattern of post-ischaemic intestinal perfusion in rats (Savas et al, 1997). Increased leukocyte/blood vessel wall interaction is only noted in the submucosa, so blood vessel plugging by marginated leukocytes is unlikely to be the cause of decreased blood flow in the muscularis layers in rats (Beuk et al, 2000). Tissue viability is proportional to patency of the microcirculation in rats (Gorey, 1980). More global measurement (^{133}Xe clearance, transit-time ultrasound) of intestinal blood flow also reveals only partial recovery after IIR in rats (Savas et al, 1997; Turnage et al, 1995b; Turnage et al, 1995a).

Experimental work aimed at clarifying the mechanisms behind the changes in splanchnic perfusion after IIR described above has focused on the changes in vascular reactivity and permeability after IIR. Mesenteric blood flow is controlled after IIR, at least in part, by endothelin acting on endothelin receptors. Endothelin-A receptors are found on smooth muscle and respond to endothelin-1 binding by vasoconstriction. Endothelin-B receptors are non-selective and ligate endothelin-1 and endothelin-3. A transient vasodilatory response is seen initially via NO release. This is followed by vasoconstriction via activation of phospholipase C, consumption of inositol triphosphate and diacylglycerol, and intra-cellular calcium concentration increase. Post-ischaemic hyperaemia is increased after endothelin-B receptor blockade (Oktar et al, 2002).

IIR results in increased mesenteric vascular permeability in rats (Ward et al, 2000; Souza et al, 2000; Turnage et al, 1994; Sun et al, 1999; Sun et al, 2002).

Increased microvascular permeability after I/R is dependent on NO and PAF in hamsters (Noel et al, 1996). It is hypothesized that I/R generates ROS that activate phospholipase A₂ that generates PAF. PAF has a short half-life but can induce local vasoconstriction, chemoattract leukocytes and increase microvascular permeability. NO may act on PAF receptors to enhance PAF-triggered responses – therefore reducing NO availability before reperfusion may indirectly reduce

microvascular permeability increases after I/R. In contrast, TNF- α does not increase microvascular permeability when superperfused into mesenteric venules despite increasing leukocyte adhesion to endothelial cells in rats (Zeng et al, 2002).

Indirect causes of IIR are also associated with alterations in splanchnic perfusion. Brief hypotension results in increased mesenteric blood flow (MBF) immediately on restoration of normotension. Normotension after prolonged hypotension does not restore baseline levels of MBF, and MBF declines further with prolonged normotension in rats (Myers and Hernandez, 1992). Severe burn followed by sepsis is followed by a significant decrease in mesenteric inflow in pigs (Tadros et al, 2003). Continuous endotoxin infusion results in decreased superior mesenteric arterial (SMA) and portal venous flow with a late fall in mesenteric vascular resistance and increase in cardiac output, restoring SMA and portal flow and mimicking the hyperdynamic phase of sepsis in sheep (Schiffer et al, 1993).

The effect of compromised intestinal blood flow on oxygen delivery to and consumption by the intestines has been examined after indirect IIR injury. Moderate haemorrhage results in reduced SMA flow and a compensatory increase in percentage oxygen extraction that maintains absolute oxygen consumption at normal levels in piglets (Crissinger and Granger, 1989). Intestinal vascular resistance is increased and blood flow reduced after IIR in very young piglets, resulting in a drop in intestinal oxygen consumption. Older piglets respond to IIR with a decrease in intestinal vascular resistance and an increase in blood flow and are able to maintain oxygen consumption (Nankervis et al, 2000). Mesenteric hypoperfusion after severe burn and sepsis results in decreased oxygen delivery to and oxygen consumption by the intestines in pigs (Tadros et al, 2003).

The dual blood supply of the liver via the portal vein and hepatic artery means that reduction of intestinal inflow will also reduce liver inflow via the portal vein. Under normal conditions, the 'hepatic arterial buffer response' (see page 3 for further details) will compensate in part for the drop in total hepatic inflow.

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After IIR the compensatory increase in hepatic arterial inflow may not occur - the loss of the 'hepatic arterial buffer response' may be due to the toxic post-IIR milieu (Turnage et al, 1996). Turnage also noted a fall in portal venous flow and in hepatic arterial inflow with prolonged sham operation only, which must be taken into consideration when planning controls for experiments of this nature.

Remote effects of IIR

IIR leads to MODS when tissue injury has been severe or the subject is particularly vulnerable, but the development of MODS is unpredictable - many patients with similar risk factors do not go on to develop MODS or develop mild, resolving forms of MODS. Some patients develop intractable MODS and, despite maximal support, die. Theoretically, the 'one-hit' model of the development of MODS states that a severe enough initial insult will result in shock and the rapid development of MODS. This version of MODS is mostly likely to be resistant to intervention and rapidly progresses to death. The 'two hit' model states that the initial insult is severe enough to trigger an inflammatory cascade but not to cause MODS. The patient or experimental animal recovers from the initial insult, but the inflammatory cascade is not well controlled, and MODS develops after a latent period. It is this latter group that is likely to be most amenable to both global and specific therapeutic intervention aimed at preventing the development and progression of MODS after IIR.

IIR results in changes in blood and lymph composition, and remote organ dysfunction with death as the final outcome in a substantial proportion of cases.

Changes in the composition of blood after IIR

IIR increases systemic haematocrit in rats (Yamamoto et al, 2001a). Rat red blood cell deformability is reduced after global low-flow - this could promote red cell plugging of capillaries and increase 'no-reflow' after reperfusion (Yamamoto et al, 2001b). Serum von Willebrand factor rises after IIR in rats (Simpson et al, 1993), possibly promoting intravascular coagulation. Leukocyte count in peripheral

blood may fall in rats (Souza et al, 2000; Squadrito et al, 1997a; Altavilla et al, 1996) or rise in dogs (Jamieson et al, 1982; Sawyer et al, 1978) and humans (Gorey and Sullivan, 1988) after IIR. Myeloid precursors in the bone marrow do not fall in rats (Squadrito et al, 1997b).

Blood oxygen content after IIR is increased by the increase in haematocrit, but oxygen consumption may not behave in a predictable way after IIR injury. Research into severe septic states (resulting in a systemic inflammatory response similar to that seen after IIR) indicates that oxygen use after sepsis is abnormal. This may be due to malfunction of mitochondria in the presence of ROS and RNOS (Singer and Brealey, 1999). This has far-reaching effects on the ability of cells to use oxygen and to dispose of ROS successfully. The ability of cells to use oxygen is inversely proportional to the severity of sepsis present. ROS are created within mitochondria as the respiratory chain is increasingly unable to use molecular oxygen. Cytokines released during sepsis and IIR also directly effect mitochondrial function. Post-sepsis oxygen transport is abnormal - no-flow capillary percentage increases, fast-flow to normal flow capillary percentage increases, and capillary venular-end oxygen saturation decreases without a change in capillary arteriolar-end oxygen saturation. Capillary oxygen extraction increases three fold, and is proportional to the degree of no-flow in rats (Ellis et al, 2002). Oxygen extraction in the intestines at critically low-flow is impaired after endotoxin exposure in dogs (Schumacker et al, 1995). Some groups suspect that a similar inability to use oxygen may occur after IIR, but it is not clear if this is due to IIR-induced sepsis or a primary effect of IIR.

Oxygen consumption after IIR induced by global low-flow states has also been studied. Hepatic, intestinal and renal inflow (but not cardiac or cerebral inflow) decrease after severe haemorrhage: oxygen extraction in the liver, intestines and kidneys also decreases significantly in rats (Ba et al, 2000). Significant mesenteric hypoperfusion followed by normoperfusion in 3 day old piglets results in a significant drop in oxygen consumption in the intestines

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(Nankervis et al, 2000). This is not due to a lack of oxygen demand but to a lack of oxygen supply due to an increase in mesenteric vascular resistance. Older piglets (35 day old) respond to IIR with a decrease in mesenteric vascular resistance, an increase in oxygen delivery and normal levels of oxygen consumption.

Other experimental conditions may impact on oxygen consumption. Arterial pH falls with IIR in rats (Yao et al, 1995) and dogs (Takeyoshi et al, 1999). Oxygen extraction changes with core temperature: whole body oxygen consumption in dogs at 34°C is 31% lower than at normothermia (38°C) and 20% higher at hyperthermia (41°C). The critically low-flow threshold (at which oxygen consumption becomes supply-dependent) is reduced to 5.6 ± 0.95 ml/minute/kg at hypothermia compared to 7.4 ± 1.2 ml/minute/kg at normothermia, but the maximum percentage oxygen extraction at hypothermia was decreased to $65 \pm 10\%$ compared to $71 \pm 10\%$ at normothermia (hyperthermia increased maximum oxygen extraction efficiency to $76 \pm 5\%$) (Schumacker et al, 1987).

Changes in the composition of lymph after IIR.

Mesenteric lymph duct ligation prevents post-haemorrhage red blood cell dysfunction in the systemic circulation in rats (Zaets et al, 2003). Interruption of mesenteric lymph recirculation is also associated with reduced lung injury after haemorrhage. Mesenteric lymph produced after haemorrhage is cytotoxic to endothelial cells, increases endothelial permeability and activates circulating leukocytes. Lactate concentration in thoracic duct lymph increases after IIR in pigs (Schlichting and Lyberg, 1995). A central role for mesenteric lymph in the genesis of MODS after haemorrhage and burns (both likely to induce IIR) with particular impact on the lungs (the first remote organ reached by mesenteric lymph on joining circulating blood) has been proposed by Deitch (Deitch, 2001).

Remote organ injury: liver

The liver is especially vulnerable to remote organ injury after IIR as the hepatocytes lining the liver sinusoids are the first cells to encounter portal blood

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after it has left the intestine and the liver as a whole undergoes substantial inflow reduction during intestinal ischaemia and in the latter stages of intestinal reperfusion. The mechanisms underlying hepatic injury after IIR are still unclear. Cell culture studies show that ischaemic intestinal epithelial cells can activate liver macrophages (Kupffer cells) via a cell-free medium, but the nature of this intercellular messenger has not been elucidated: it does not appear to be either TNF- α , IL-6 or endotoxin (Towfigh et al, 2000). Kupffer cell inactivation in mice reduces the number of non-perfused sinusoids and leukocyte adhesion observed after IIR injury. It therefore appears that Kupffer cells in some way contribute to hepatic injury after IIR (Horie et al, 1997c).

The flow of blood through the liver is compromised during IIR: a 75% reduction in liver surface blood flow is seen during intestinal ischaemia in mice (measured using a laser Doppler flow probe on the surface of the liver) (Horie et al, 1998b). This expected drop in liver blood flow during intestinal ischaemia is followed by a partial recovery on intestinal reperfusion, but this is not sustained in rats (Turnage et al, 1996). The 'hepatic arterial buffer response' is not observed, and hepatic arterial inflow is also severely depressed during intestinal reperfusion. Portal venous pressure increases after IIR and liver tissue flow recovers to 80% baseline in dogs, which is lower than expected, given final total hepatic inflow of 85% of baseline (Nakamura et al, 2001). Global hypo-perfusion has also been shown to compromise liver inflow. Severe burn results in decreased hepatic arterial with increased portal venous flow; subsequent intravenous LPS (mimicking post-burn sepsis) results in decreases in both hepatic arterial and portal venous flow. The 'hepatic arterial buffer response' is lost. A hyperaemic phase is observed after prolonged LPS exposure in pigs, and portal venous pressure rises (Tadros et al, 2000).

Leukocytes are sequestered in liver sinusoids and a proportion of sinusoids become non-perfused after IIR. Adhesion and sequestration are selectin-dependent (liver sinusoid leukocyte sequestration after IIR is equivalent to

baseline in P-selectin -/- knockout mice) but rolling is selectin-independent and may be integrin-dependent (Kubes et al, 2002). Leukocytes accumulate in both midzonal and pericentral areas of sinusoids, and NADH autofluorescence (indicating oxidative stress) increases most markedly in pericentral areas. NADH autofluorescence correlates positively with the number of non-perfused sinusoids [$r = 0.750$] and the number of stationary leukocytes [$r = 0.748$]. There was also positive correlation between the number of stationary leukocytes and the percentage of non-perfused sinusoids [$r = 0.897$]. The number of non-perfused sinusoids increases 10-fold with IIR in rats (Horie et al, 1996; Yamagishi et al, 2002). An increased effect of IIR on non-perfusion of hepatic sinusoids, leukocyte adhesion and NADH autofluorescence by NOS inhibition with L-NMMA (NG-monomethyl-L-arginine), and a decreased effect with L-arginine and diethylenetriamine (NO donors) in mice may shed some light on the mechanism underlying IIR-induced hepatic injury (Horie et al, 1997b). P-selectin expression is increased in the liver (and intestine and lung) in line with increased leukostasis after IIR injury in mice (Horie et al, 1998a). P-selectin expression and leukostasis are enhanced by NOS inhibition with L-NMMA and reduced by NO donation by L-arginine and diethylenetriamine-NO. P-selectin, ICAM-1 or CD11/CD18 deficient mice have fewer non-perfused sinusoids, stationary leukocytes and less NADH autofluorescence than wild-type mice after IIR (Horie et al, 1997a). As well as sequestering leukocytes, liver tissue becomes more permeable after IIR: hepatic capillary permeability to ^{125}I -labelled albumin increases after IIR only if reperfusion is permitted in rats (Poggetti et al, 1992b; Sun et al, 2002).

Liver metabolism is adversely affected by IIR. Bile production is an energy-dependent process, relying on sodium/potassium ATP-ase exchangers – a reduction in bile production is used as a surrogate marker of hepatic energy depletion. Despite ensuring a uniform delivery of oxygen to the liver, bile production by the liver falls in rats with increasing intestinal reperfusion time in previously IIR injured animals compared to previously sham operated animals

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(Poggetti et al, 1992a), implying that the reduction in bile production after IIR is not dependent on the supply of oxygen. No change in bile flow rate is noted with sham operation or intestinal ischaemia only – a drop in bile flow rate is initiated soon after intestinal reperfusion and becomes more marked throughout reperfusion in rats (Turnage et al, 1994; Turnage et al, 1996) and dogs (Nakamura et al, 2001). Bile salts neutralize LPS. If the liver fails to generate bile after IIR, LPS neutralization will be compromised making LPS escape into the systemic circulation and subsequent endotoxaemia more likely (Koike et al, 1994).

Hepatic ATP levels decrease (Turnage et al, 1996) and hepatic ADP and Pi levels rise (Changani et al, 1998) in rats after IIR, but only after the onset of intestinal reperfusion. IIR is only associated with hepatic energy failure (fall in ATP, rise in Pi), and death if performed at normothermia in rats (Vejchapipat et al, 2001). Hepatic energy failure is delayed if IIR is inflicted at moderate hypothermia (32°C). Further experiments by Vejchapipat (Vejchapipat, 2001) examined the metabolic effect of IIR on the rat liver in greater detail. Hepatic lactate, succinate, glutamate and alanine increase with intestinal reperfusion and hepatic glutamine decreases. No change is found in hepatic glucose. Endotoxaemic pigs experience a net hepatic consumption of lactate (Bathe et al, 1998b), and isolated perfused dog livers switch from lactate extraction to production at low hepatic inflow rates (Samsel et al, 1991). The ketone body ratio of acetoacetate to 3-hydroxybutyrate, which reflects hepatic mitochondrial redox status, only falls after intestinal reperfusion is permitted in rats (Poggetti et al, 1992b) and dogs (Nakamura et al, 2001). Hepatic hypoperfusion induces the rapid development of hepatic insulin resistance with profound hyperinsulinaemia and hyperglycaemia in rats (Ma et al, 2003). IIR results in an increase in oxidised glutathione levels in liver tissue, but no change in total glutathione levels in rats (Turnage et al, 1991). Turnage also found no change in hepatic lipid peroxidation products (MDA tested via TBARS). Hepatic superoxide dismutase, nitric oxide and MDA all increased after IIR in rats (Fu et al, 1997). Oxygen handling by the liver is altered by IIR. Hepatic venous

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oxygen saturation falls from 90% to 50% after SMA occlusion for 120 minutes followed by reperfusion for 120 minutes in dogs (Takeyoshi et al, 1999).

Hepatic drug metabolism, an alternative measure of liver function, is also compromised after IIR. Antipyrine clearance is reduced after IIR injury without change in apparent volume of distribution or binding in dogs, indicating an adverse effect on hepatic oxidative drug metabolism (Gurley et al, 1997).

Evidence of loss of liver enzymes into systemic blood indicates hepatocyte disruption after IIR. Increased serum aspartate transaminase [AST] (Yamamoto et al, 2001a; Akcakaya et al, 2002) and alanine transaminase [ALT] (Fu et al, 1997; Horie et al, 1998b; Horie et al, 2002; Yamagishi et al, 2002; Turnage et al, 1996) have been noted after IIR. IIR results in increased serum glutamine pyruvic transaminase in rats (Simpson et al, 1993). The rat liver has been found to have histological damage after IIR (Fu et al, 1997). Electron microscopy of the rat liver after intestinal ischaemia reveals Kupffer cell activation and blood stasis within liver sinusoids. Intestinal reperfusion results in mitochondrial swelling, dilatation of the rough endoplasmic reticulum and cytoplasmic lipid droplets within hepatocytes (Savas et al, 2003). The liver also has a higher neutrophil count per 50 high-power fields after IIR in rats (Simpson et al, 1993).

Remote organ injury: lung

The lung is vulnerable to injury after IIR. Lung high energy molecules are depleted: whole lung ATP levels were reduced after IIR in rats (Schmeling et al, 1989) and cultured pulmonary artery endothelial cells suffer ATP depletion after exposure to plasma from IIR-treated animals (Gerkin et al, 1993).

Neutrophils are sequestered in the lung after IIR – this may be quantitated by measuring levels of MPO contained predominantly within neutrophils, or by histological examination. Lung MPO levels rise after IIR (Lane et al, 1997; Squadrito et al, 1997b; Tullis et al, 1996; Schmeling et al, 1989; Simpson et al, 1993; Dowdall et al, 2002). Lung histology shows that IIR injury in rats results in pulmonary congestion and inflammatory cell infiltration (Wada et al, 2001).

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Pulmonary macrophage expression of pulmonary MIP-2 mRNA increases after IIR in mice (Zhao et al, 2002). IIR injury-primed circulating neutrophils provoke lung injury in rats – an effect that is abolished by depleting neutrophil numbers or blocking neutrophil activation. This effect is independent of the presence of LPS (Koike et al, 1994). Further evidence that neutrophils primed by IIR injury are involved in IIR-related lung injury is provided by isolated lung studies. Rat lungs perfused with cell-free perfusate or neutrophils from IIR injured animals increase in wet-to-dry weight ratio, increase pulmonary artery pressure and ultrastructural damage occurs only if exposed to IIR-injured neutrophils (Kadesky et al, 1995).

Lung capillaries become more permeable after IIR (Souza et al, 2000; Poggetti et al, 1992b; Turnage et al, 1994; Tullis et al, 1996; Schmeling et al, 1989; Ohara et al, 2001; Ward et al, 2000; Sun et al, 2002; Simpson et al, 1993; Dowdall et al, 2002; Iglesias et al, 1998a). One of the reasons for increased vascular permeability and leukocyte sequestration in the lung after IIR may be increased expression of adhesion molecules on the lung endothelium – for example ICAM-1 has been shown to rise after IIR in mice (Zhao et al, 2002). Bioactive molecules released after IIR may also have direct effect on lung tissue. Acute endotoxaemia activates both alveolar macrophages and epithelial cells. The latter may contribute to acute pulmonary inflammation in the presence of endotoxin in rats (Sunil et al, 2002). TNF- α released by the rat liver after I/R is associated with an increase in pulmonary capillary permeability: IIR will result in an element of hepatic I/R, and it is possible that this effect is pertinent to the lung injury seen after IIR (Colletti et al, 1990).

It is suggested that the particular vulnerability of the lungs to ROI following IIR may be related to toxic factors delivered to the lungs via mesenteric lymph. Much of the work relating to this has used animal models of haemorrhage or burns in which IIR is expected. The nature of the suggested toxic factors is not clear at present but they do not appear to be cytokines in rats (Davidson et al, 2004).

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Ligation of mesenteric lymphatics protects against lung injury after haemorrhagic shock in rats (Sambol et al, 2000).

Remote organ injury: cardiovascular system

IIR has profound effects on cardiovascular stability - mean arterial blood pressure (MAP) falls, heart rate rises, peripheral resistance falls. This response to IIR will hamper maintenance of mesenteric blood flow during intestinal reperfusion (Takeyoshi et al, 1999; Squadrito et al, 1997a; Yao et al, 1996; Squadrito et al, 1997b; Bathe et al, 1998a; Altavilla et al, 1996; Aneman et al, 1996; Gathiram et al, 1989; Schmeling et al, 1989; Yao et al, 1995; Nakamura et al, 2001; Hayward and Lefer, 1999; Park et al, 1990; Myers and Hernandez, 1992; Khanna et al, 2001).

Cardiac function is impaired [reduced left ventricular isovolaemic pressure, slowed rate of left ventricular contraction and relaxation, reduced response to increasing the concentration of calcium ions in the perfusate and impaired response to increasing the coronary blood flow rate] following IIR injury in rats despite no gross peripheral cardiovascular dysfunction (Horton and White, 1993). Rat heart damage after IIR has been shown histologically (Yao et al, 1996) and cardiomyocyte damage is implied as serum levels of MB fraction of creatine kinase increase after IIR (Akçakaya et al, 2002).

IIR has a global effect on vascular reactivity. IIR causes aortic ring hyporeactivity when stimulated with phenylephrine and acetyl choline, and is associated with a rise in ICAM-1 expression in the aortic wall (Squadrito et al, 1997a) - this change is not related to the presence or absence of endothelium on the aortic rings (Squadrito et al, 1997b). Vascular reactivity is proportional to the production of ROS by endothelial cells, and the subsequent production of eicosanoids (6-keto-prostaglandin- $F_{1\alpha}$, prostaglandin- $F_{2\alpha}$, prostaglandin- E_2 and thromboxane B_2) but is not affected by the presence of NO (because endothelium-denuded vascular rings showed no change in the post I/R pattern of reactivity). At low levels of ROS production, vascular rings demonstrate reduced responsiveness to noradrenaline (but not adrenaline), generating less tension (i.e.

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showing less vasoconstriction) when exposed to noradrenaline after I/R. This may be due to lower affinity of noradrenaline to receptors after a conformational change in catecholamine receptors after I/R or to an alteration in signal transduction within the cell after I/R. At higher levels of ROS production, increased eicosanoid production causes marked vasoconstriction in post I/R vascular rings (Benoit and Taylor, 1997).

IIR results in significant and sustained mesenteric vasoconstriction. Loss of endothelium-dependent microvascular relaxation after IIR blunts relaxation of superior mesenteric arterial rings when stimulated with acetyl choline (Wada et al, 2001; Hayward and Lefer, 1999). Mesenteric arterial ring dose-response to endothelin-1 is altered after IIR implying excessive vasoconstriction due to endothelin-A receptor activation and/or endothelin-B receptor blockade, and agonist induced NO production by mesenteric arterial rings after IIR is very much reduced (Nankervis et al, 2000). Synthesising these results, it is hypothesised that after IIR injury endothelin-A receptors have greater affinity for endothelin-1, in part due to inactivation of endothelin-B receptors. The vasoconstricting action of endothelin-A receptors is therefore largely unopposed, an effect which is exaggerated by the loss of NO release from endothelium after IIR which would normally form part of the local vasodilatation mechanism. Endothelin-1 may also act directly to close pre-capillary sphincters thus shunting blood away from mesenteric capillaries. This will increase the drop in mesenteric oxygen consumption as oxygen cannot reach intestinal cells and mesenteric venous oxygen content rises due to non-perfusion of the capillary bed. The drop in mesenteric oxygen consumption is not due to a drop in tissue oxygen demand (as is seen after moderate hypothermia) but a critical drop in oxygen supply, most likely to be mediated by abnormal endovascular responses to endothelin-1.

Expression of adhesion molecules by vascular endothelium both locally and systemically is promoted by IIR. ICAM-1 mRNA expression in mesenteric vascular tissue is increased after IIR in rats (Wada et al, 2001). Vascular cell

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adhesion molecule-1 (VCAM-1) expression in rat aortic endothelium is increased after IIR (Altavilla et al, 1996). PMNs demonstrate increased adherence to superior mesenteric artery rings after IIR (Hayward and Lefer, 1999). Septic shock (a global low-flow state) increases membrane expression (but not cytosolic levels) of G-protein-coupled receptor kinase-2. These receptors phosphorylate agonist-occupied receptors thus locking them permanently on or off and removing their ability to respond further to signalling arising outside the cell (Kadoi et al, 2002). This may contribute to the loss of response to β -adrenergic stimulation seen with severe sepsis.

Some investigators have used IIR injury to 'prime' the cardiovascular system to withstand a subsequent direct insult. A brief IIR injury reduces the magnitude of cardiac injury after coronary artery occlusion in rats – an example of the 'priming effect' working on a remote organ (Wang et al, 2001). Inhibition of inducible NOS abolishes the beneficial effect of intestinal priming on myocardial infarct size and limits the reduction in MPO activity after myocardial I/R. No effect on non-primed myocardial I/R injury was seen. Wang hypothesises that although intestinal IIR injury does not increase iNOS in myocardium before myocardial ischaemia, after 30 minutes of myocardial ischaemia, infarcted areas and areas at risk of infarction do express more iNOS. Therefore, during myocardial reperfusion, local NO production may be boosted by this newly made iNOS. This may limit local neutrophil infiltration, prevent cell calcium overload, reduce catecholamine-induced increases in myocardial activity (and therefore limit oxygen demand and ATP use by myocytes after I/R), increase glycolysis (thus improving ATP generation) and locally dilate the coronary microvasculature (reducing local ischaemia).

Remote organ injury: kidneys

Patients suffering IIR injury often experience renal insufficiency if multiple organ dysfunction develops. Some experimental work has examined the effect of IIR on the kidneys. IIR reduces renal blood flow by more than 80% compared to

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pre-IIR baseline in rats. Renal ATP levels are reduced by 25% and fractional excretion of sodium is increased after IIR indicating renal tubular dysfunction (LaNoue et al, 1996). This reveals a significant impact of IIR injury on renal function, possibly related to the severe restriction on renal inflow after IIR. IIR reduces renal blood flow, inulin and sodium clearance in rats. Eicosanoid release is increased with proportionately more thromboxane A₂ released than prostaglandins E₂ and 6-keto-prostaglandin F_{1α} (Rothenbach et al, 1997). Rothenbach et al related some of the effects of IIR injury on the kidneys to ROS, as pre-treatment with pentoxifylline and allopurinol preserves renal function and reduces eicosanoid release.

Mortality of IIR

Experimental studies designed to report mortality rates after IIR injury show high percentage mortality: 50 - 100% (Yamamoto et al, 2001a; Yagihashi et al, 1998; Yao et al, 1996; Squadrito et al, 1997b; Altavilla et al, 1996; Zingarelli et al, 2001; Ohara et al, 2001; Yamada et al, 1995; Hayward and Lefer, 1999; Pitt et al, 1991; Schleiffer and Raul, 1996). The variation within these reports of mortality after IIR may be explained by species, severity of insult and experimental technique. Clinical reports of mortality after mesenteric ischaemia are also high: 75% (Clavien et al, 1987); 92% (Wilson et al, 1987); 71% (Gorey and Sullivan, 1988). After revascularisation for acute mesenteric ischaemia, reported 30 day mortality falls to 43% (Bjorck et al, 2002), which is likely to reflect the relative fitness of patients selected for revascularisation. Revascularisation for chronic mesenteric ischaemia has a lower reported mortality rate – 28% in a small series reported by Harward (Harward et al, 1993) – that is likely to reflect the prior establishment of collateral supply to the intestines and lack of acute, severe intestinal ischaemia.

The intestine as a remote organ affected by I/R in other organs

Vascular surgery often also involves reductions in blood flow to regions other than the splanchnic bed during operative intervention. It has been noted that prolonged ischaemia of the lower limbs (for example during aortic or reconstructive surgery) may be followed by impaired intestinal function on lower limb reperfusion, with increased permeability to endotoxin and (in some studies) bacteria. Morphological changes in the intestine on lower limb reperfusion have also been described. This is an example of remote organ injury after ischaemia-reperfusion in affecting the intestines. Experimental evidence suggests that the intestine may be uniquely vulnerable to remote effects of ischaemia-reperfusion (I/R) injury. Reperfusion after unilateral hind limb ischaemia with both arterial and venous occlusion in rats did not result in abnormal microvascular responses in the toe skin of the contra lateral limb. No increased leukocyte rolling or adhesion was seen in that microvascular bed, and histology of the skin was normal (Dammers et al, 2001). Rat splanchnic microvascular flow was reduced in both arteries and veins with reperfusion after unilateral hind limb arterial and venous ischaemia (Wehrens et al, 2002). Bilateral hind limb ischaemia-reperfusion increases leukocyte interaction with mesenteric post-capillary venules in rats (Sobral et al, 1999). Ischaemia alone increases leukocyte rolling only, but reperfusion increases rolling and adhesion. Subtle histological changes are found in small bowel morphology with impaired small bowel barrier function after bilateral hind-limb I/R in rats. Large bowel structure and function is preserved (Yassin et al, 1997). Bilateral hind limb arterial and venous ischaemia also results in endotoxaemia and the consumption of anti-endotoxin antibodies in rats, but not in bacterial translocation (Yassin et al, 1998).

Age and IIR

There is a paucity of data dealing directly with the differences between adults/mature animals and children or infants/immature animals in the response to IIR. It may be anticipated that younger animals and patients respond differently to an IIR insult because it is thought that patients groups of different ages respond to serious illness in disparate ways. For example, there are subtle differences in the pattern and severity of MODS experienced by adult and paediatric patients during severe sepsis. Adult severely septic patients experience severe cardiovascular compromise, followed by pulmonary, renal and hepatic impairment (Brun et al, 1995); a complicated post-operative course in surgical patients is also often heralded by cardiovascular compromise first, followed by respiratory and renal insufficiency, then hepatic and haematological failure (Oshima et al, 2000; Bush et al, 1995). Severely septic paediatric patients are noted to develop sequential respiratory, hepatic and renal failure (Doughty et al, 1998a), and infants with severe necrotizing enterocolitis most frequently experience respiratory, renal, cardiovascular and hepatic failure (Morecroft et al, 1994). Similar effects are seen with elderly patients – smaller insults result in more severe metabolic and physiological disturbance as physical reserve is eroded by age and co-morbidity. Certain conditions thought to have a significant IIR-related element predominantly affect the premature infant: for example, NEC. In part these age-related differences may be because metabolic pathways and physiological reserve of the young animal or patient continue to mature/enlarge after birth, and therefore the fate of toxins and metabolites released after severe insults vary with the age or maturity of the patient or animal.

A few groups have attempted to explore the age-related effects of IIR injury using animal models. 10-day old rats show increased intestinal permeability to ⁵¹Cr EDTA with a variety of insults (IIR, hypoxia and high-dose intraperitoneal indomethacin) (Langer et al, 1993a). Sustained hypoperfusion of the intestine followed by normoperfusion in 3- and 35-day old piglets shows that younger

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piglets experience more disruption after IIR to their vascular response to endothelin-1 (mesenteric arterial rings are unable to relax) and to NO agonists (mesenteric arterial rings are unable to produce NO after IIR). Mesenteric vascular resistance is increased and both delivery and consumption of oxygen in the mesenteric region are reduced after IIR. Older piglets show similar endovascular dysfunction, but the effects of IIR are less marked - mesenteric vascular resistance is reduced, oxygen delivery to the mesenteric region is increased but the level of oxygen consumption does not change (Nankervis and Nowicki, 2000). Global low-flow (induced by moderate haemorrhage) results in reduced SMA flow in 1-, 3-, 7-, and 14-day old piglets and a compensatory increase in percentage oxygen extraction that maintains absolute oxygen consumption at normal levels. After severe haemorrhage, only 14-day old piglets can increase percentage oxygen extraction sufficiently to maintain oxygen consumption in the face of a more severe drop in SMA flow (Crissinger and Granger, 1989). A differential effect of intra-luminal lipids is found on intestinal permeability increases after IIR. 1-day old pigs suffer greater increase in intestinal permeability if non-delipidated formula feed, lipid or monounsaturated fatty acids are present intra-luminally – an effect that is not seen in 1-month old pigs. Intra-luminal delipidated formula feed is not associated with increased intestinal permeability with IIR in 1-day old pigs. It is of interest that 1-day old pigs show increased intestinal permeability without intestinal ischaemia or reperfusion with intra-luminal lipid and monounsaturated fatty acid (Crissinger and Tso, 1992). Crissinger hypothesizes that intra-luminal fatty acids may be involved in the increased susceptibility of fed neonates to NEC, and this may allow a lipid-elimination therapeutic strategy for the prevention of NEC in high-risk neonates. The effect of low birth weight, a key feature of prematurity, on the spontaneous appearance of NEC-like lesions has been explored using low birth weight piglets (Sibbons P.D., 1990; Thornbury et al, 1993).

Hypothermia and IIR

Mechanism of protective effects

Hypothermia has been used as an experimental and clinical intervention to abrogate the effects of systemic inflammation and local tissue damage including IIR injury for some time. The mechanisms behind the protective effects of hypothermia are not clear but are thought to involve a combination of reduced metabolic rate, reduced energy depletion, reduced alteration in ion flux, reduced vascular permeability and reduced tissue oedema (Barone et al, 1997) and possibly also induction of the HSR and altered oxygen consumption.

When exposed to hypoxia during hypothermia, brain slice ATP levels are preserved. Slices at 34°C are able to tolerate 10 minutes of hypoxia without loss of ATP, 58% of neurons recover resting and action potentials, neuronal membrane potential is preserved and increases in intra-cellular calcium levels are attenuated. These protective effects are lost if local glucose concentration is reduced. Brain slices at 37°C fail to recover membrane, resting or action potentials, lose ATP and suffer calcium influx (Wang et al, 2000). The mechanism underlying hypothermic protection of myocardium during ischaemic arrest may involve inhibition of apoptosis. Functional cardiac recovery is superior and mRNA expression of mitochondrial proteins, adenine translocase and the β -subunit of F1-ATPase are preserved after ischaemia at 30°C. cDNA array analysis of rabbit hearts reveals an effect of ischaemia at 34°C on 13 genes and a modification of this effect with ischaemia at 30°C for 8 genes – 6 of which are related to apoptosis (Ning et al, 2002). The mechanism underlying hepatic protection by hypothermia during hepatic ischaemia has been explored in mice: hepatic tissue injury, serum transaminase, TNF- α , IL-1 β and MIP-2 levels are reduced in hypothermic animals after liver I/R (Kato et al, 2002). Activation of transcription factor NF κ B was not reduced, but AP-1 and c-jun NH₂-terminal kinase were both greatly attenuated.

Whole body hypothermia induces the HSR with increased expression of heat shock protein (HSP) 72 mRNA in the brain, heart, kidney, liver and lung

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mediated by heat shock factor-1 (HSF-1). HSF-1 also mediates the HSR to hyperthermia, but the mechanism by which hypothermia activates HSF-1 is different in that it does not cause hyperphosphorylation of HSF-1 (Cullen and Sarge, 1997). Exposure of rats to an ambient temperature of 6°C results in the induction of HSPs in the HSP70 family in brown adipose tissue and in the aorta. Increased HSP expression is blocked by sympathetic ganglionic blockade and by α -1 adrenergic blockade with prazosin (Matz et al, 1996). Oxygen extraction at hypothermia (34°C) compared to normothermia (38°C) and hyperthermia (41°C) during step-wise haemorrhage in dogs is associated with significant decreases in whole body oxygen extraction, critical oxygen delivery level and critical extraction ratio (Schumacker et al, 1987).

Experimental applications of hypothermia

Hypothermia has been used experimentally to reduce the local impact of IIR, liver I/R, vascular reactivity, response to cytokines and endotoxin, haemorrhagic shock and myocardial infarction.

IIR at hypothermia (30 -32°C) in rats results in zero increase in leukocyte adhesion and decrease in mucosal blood flow compared to animals at normothermia (36 - 38°C). Increases in macromolecular leak from villi were seen, but leak was less marked in hypothermic animals (Kalia et al, 2002). Cat intestinal permeability rises after normothermic IIR, but not after hypothermia alone or hypothermic IIR (Jurkovich et al, 1988). Hypothermic intestinal ischaemia followed by normothermic reperfusion resulted in greater intestinal permeability than normothermic IIR, but identical flow rates during reperfusion compared to hypothermia throughout IIR in rats. Hypothermia late in intestinal ischaemia and during early reperfusion reduces increases in intestinal permeability and results in less histological damage to the intestine compared to intestine maintained at normothermia throughout IIR (Udassin et al, 1997). There is no local benefit for intestinal metabolism after hypothermic IIR in rats (Vejchapipat et al, 2001).

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A decrease in rat hepatic ATP after shock was prevented by maintenance at 28°C - ATP also recovered to significantly higher levels on reperfusion after shock and local hepatic ischaemia at 28°C compared to normothermic shock (Johannigman et al, 1992). Topical hepatic hypothermia (cooling the liver only to 25°C) reduced the sequelae of partial hepatic ischaemia followed by reperfusion both locally and in the lung in rats. Locally hypothermic animals exhibited less hepatic necrosis, less hepatic PMN infiltration, lower peak systemic serum TNF- α levels and less lung injury (lower MPO activity and less Evans blue extravasation) than normothermic animals (Patel et al, 2000).

Cooling rabbit mesenteric artery and aorta to 28°C in an organ bath increased the potency of noradrenaline in producing vasoconstriction in the mesenteric artery but decreased its potency in the aorta. Maximum vascular response to histamine by the mesenteric artery was attenuated by cooling to 28°C, but responsiveness to noradrenaline was not changed by cooling (Allen et al, 1996).

Cooling human umbilical vein cultured cells to 25°C inhibited the transcription and expression of E-selectin and tissue factor after stimulation with TNF- α and IL-1; this recovered on rewarming the cells to 37°C. Induction of E-selectin and tissue factor was not attenuated by hypothermia (Johnson et al, 1995).

Prolonged hypothermia (34°C) during and after haemorrhagic shock followed by shed-blood and Ringer's lactate resuscitation in rats significantly improved survival time and survival rate compared to normothermic (38°C) shock (Prueckner et al, 2001). Maintaining core temperature at 34°C during lethal uncontrolled haemorrhagic shock in rats resulted in a doubling of survival time (Takasu et al, 1999). Whole body hypothermia (32.5 – 33°C) after haemorrhagic shock in rats reduced IL-6, ROS, AST, α GST (alpha-glutathione S-transferase) and creatinine release, but no decrease was noted in TNF- α or IL-10 release (Gundersen et al, 2001). Unresuscitated haemorrhagic shock in pigs followed by hypothermia (32.5°C) before rewarming to normothermia resulted in decreased systemic oxygen extraction, slowed coagulation (but normal clot strength) and fibrinolysis during

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hypothermia. Rewarming restored all these values to normal levels (Heinius et al, 2002). Normothermic shock to a mean arterial pressure of 30 – 40 mmHg in dogs can be followed by prolonged deep hypothermic circulatory arrest (DHCA: core temperature of 10°C, induced and reversed with cardio-pulmonary bypass) for 1 hour without induction of MODS or neurological deficit on recovery (Capone et al, 1996). Capone et al proposed that DHCA may allow bloodless field operating to salvage multiply injured patients even after severe haemorrhagic shock. Cardio-pulmonary bypass at 18°C for 1 hour of circulatory arrest then reperfusion at 37°C in pigs results in impaired vasodilatation in cerebral, renal and pulmonary vessels and increased duodenal apoptosis (Cooper et al, 2000). It was hypothesized that DHCA induces endothelial dysfunction in several vascular beds and permitted apoptotic cell death in the intestines that may indicate the pathogenesis of MODS after DHCA.

Endovascular cooling to reduce core temperature to 34°C during left anterior descending artery occlusion in pigs significantly reduced infarct size compared to normothermia. Hypothermic animals had a reduced heart rate but increased stroke volume, thus maintaining cardiac output (Dae et al, 2002).

Hypothermia may have different effects on the mature and immature animal. Cold stress (32°C for 1 hour) followed by rewarming caused a decrease in heart rate and cardiac output in premature (7 – 10 days premature), newborn (1 – 2 days old) and neonatal (1 – 2 weeks old) piglets. Newborn and neonatal piglets had significantly reduced cerebral and intestinal blood flow during hypothermia; the latter persisted after rewarming. Cardiac output failed to return to baseline in any age group (Powell et al, 1999).

Remote organ injury and hypothermia

Hypothermia has also been used to protect remote organs from injury after IIR. Hypothermia (30 - 33°C) during IIR protected against death and delayed the onset of hepatic energy failure (increase in the ratio of hepatic Pi to ATP measured *in vivo* by magnetic resonance spectroscopy [MRS]) after IIR injury in rats, but did

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not protect against intestinal energy failure (Vejchapipat et al, 2001). Rat hepatic ATP levels were also protected by moderate hypothermia during gradual hypoxia (Takahashi et al, 2004).

Hypothermia and transplantation

Cold-storage of donor organs is a form of hypothermic treatment that has been used since the early development of organ transplantation. Re-implantation of cold-stored and normothermically reperfused auto-transplanted intestinal segments in dogs resulted in a rapid decline in blood flow during reperfusion, and reduced intestinal oxygen consumption compared to control (non-transplanted) segments. MPO activity increased in reperfused segments, LTB₄ and NO synthesis by the mucosa increased, and histological damage was seen (villous epithelial loss, decreased villous height, venous congestion) (Mangino et al, 1996). In studies of this kind it is difficult to establish which adverse effects are related to hypothermic organ storage and which are related to IIR injury. The human colon is less susceptible to cold preservation injury than human ileum: it retained more electrophysiological function and response to theophylline stimulation compared to the ileum (Kawashima et al, 1999).

Hypothermia and brain preservation

Hypothermia has been used experimentally and clinically to reduce the impact of brain injury of several types: traumatic, ischaemic and hypoxic. Focal cooling of the skull to 32 -34°C in rats reduced cerebral infarct size after middle cerebral artery occlusion and reperfusion: cooling the brain to 29°C during ischaemia completely prevented brain infarction (Barone et al, 1997). 3 hours of hypothermia (30°C) initiated 5 minutes after normothermic traumatic brain injury in rats reduced the number of necrotic neurones and contusion volume 3 days after injury (Dietrich et al, 1994). Whole body hypothermia (33°C) in rats was associated with a reduction in size of the ischaemic area during middle cerebral artery occlusion compared to maintenance at normothermia (37.5°C). c-fos and HSP70

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mRNA expression in brain tissue was also reduced in the hypothermic group (Mancuso et al, 2000).

Clinical applications of hypothermia

Hypothermia has been used clinically to help infants at risk of hypoxic brain damage and adults after ischaemic stroke, traumatic brain injury, liver failure, severely sepsis and acute respiratory distress syndrome (ARDS) with mixed results. Inadvertent hypothermia has also been studied after sepsis, surgery and trauma.

Gluckman et al (Gluckman et al, 2004) has recently reported a randomised trial of 'cold-cap' therapy (water-cooled hat designed to lower core cerebral temperature for 72 hours) for neonates at high risk of hypoxic brain damage, and demonstrated a better functional neurological outcome in neonates treated with 'cold-caps' within 6 hours of birth. Whole body hypothermia (to a core temperature of 33°C) for 48 to 72 hours within 14 hours of severe ischaemic stroke of the middle cerebral artery was tested in a series of 25 patients. After 48 to 72 hours all patients were rewarmed to 37°C. 11/25 patients died due to cerebral herniation during rewarming - the remaining 14 patients survived. 10 patients suffered pneumonia. Outcome at 4 and 13 weeks was assessed and neurological outcome was above average given the severity of the stroke in all hypothermic patients (Schwab et al, 1998). A randomised, controlled trial of whole body hypothermia (33°C) for closed acute traumatic brain injury recruited 392 patients. Target hypothermic temperature was achieved after 8 hours. No difference in death rate or in poor outcome (severe disability, persistent vegetative state or death) was demonstrated, even though fewer hypothermic patients had high intracranial pressures than normothermic patients. Hypothermic patients had longer hospital stays (Clifton et al, 2001). Whole body hypothermia (32 - 33°C) reduced intracranial hypertension in patients with acute liver failure awaiting transplant. Arterial ammonia and cerebral uptake of ammonia were reduced during hypothermia, and no adverse effects of hypothermia were observed (Jalan et al,

1999). A small clinical trial of hypothermia (32 - 35°C) as an adjunct in end-stage septic ARDS found that hypothermic patients had an increased survival rate (33% *versus* 0%), reduced heart rate and reduced cardiac index compared to normothermic patients. Absolute oxygen consumption was not altered by hypothermia, but percentage extraction increased (Villar and Slutsky, 1993).

Some studies have reported inadvertent or primary hypothermia associated with disease states. Patients with severe sepsis who were hypothermic (temperature less than 34.5°C) on admission to an intensive care unit (ICU) had higher levels of systemic serum TNF- α and IL-6, higher urinary levels of metabolites of thromboxane A₂ and prostacyclin, and a higher mortality rate (Arons et al, 1999). Retrospective examination of the incidence of hypothermia (core temperature less than 34.5°C) in patients during AAA repair found that women were more likely to be hypothermic; hypothermic patients had higher APACHE (acute physiology and chronic health evaluation) II and III scores, greater intra-venous fluid, blood transfusion, vasopressor and inotrope requirements; were more at risk of MODS and death; and survivors stayed on the ICU and in hospital for longer (Bush et al, 1995). Bush et al recommended that temperature should be tightly controlled in AAA repair and maintained close to normothermia to avoid these adverse events. Of 13 cases of deep hypothermic circulatory arrest (14 - 19°C) for repair of interrupted aortic arch in infants, 3/13 infants died post-operatively – 2 from sepsis and 1 from MODS. The remaining 10 were followed up for 1 - 29 months and had no functional cardiac or neurological deficits (Tlaskal et al, 1997). This was not a case-control study, but some degree of neurological impairment is expected after circulatory arrest – the absence of neurological impairment in this study is interesting. The short follow-up period may contribute to this. Of 11 cases of thoraco-abdominal aortic aneurysm repair using cardio-pulmonary bypass, selective visceral perfusion and hypothermia (33°C), 2/11 patients died (one from an old empyema, one from MODS) and no patients suffered paraplegia (Yamashita et al, 1998). Plasma ATP levels were

transiently depressed in elective surgical patients whether operations were performed at normothermia (limb vascular surgery) or moderate hypothermia [31°C] (cardio-pulmonary bypass for coronary artery bypass surgery). Trauma patients with a core temperature of greater than 34°C and an injury severity score of less than 20 had plasma ATP levels similar to elective surgical patients. However, trauma patients with core temperatures less than 34°C and injury severity scores greater than 30 were admitted with low levels of plasma ATP that failed to recover after 24 hours (Seekamp et al, 1999).

Heat shock proteins and IIR

Novel approaches to the prevention of serious systemic sequelae after IIR are constantly sought. The HSR is a mysterious response originally noted after a brief period of hyperthermia. HSPs are believed to be molecular chaperones and expression is upregulated in the HSR. This conveys a degree of resistance to some inflammatory processes by a mechanism as yet not understood, but which may offer some protection against IIR-induced injury.

Whole body hyperthermia induces HSP expression, increases total body oxygen consumption, raises metabolic rate and lowers splanchnic blood flow in rats. Specifically: arterial oxygen saturation increases and splanchnic arterio-venous difference in oxygen carriage increases, indicating greater oxygen consumption by the splanchnic organs. Venous partial pressure of carbon dioxide increases and oxygen saturation and pH decreases. After one hour of normothermia, liver glycogen is 80% of pre-hyperthermia values, and two hours after restoration of normothermia, splanchnic arterio-venous oxygen carriage difference remains elevated (Hall et al, 1999). Hall et al speculated that these effects are either caused by hypoxic stress to the liver and intestines when hyperthermic or by generation of reactive oxygen and nitrite species after changes to cellular energy balance when hyperthermic. Intra-arterial ³H-misonidazole (a marker of cell hypoxia) followed by liver and small bowel biopsies showed an 80%

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increase in uptake in the liver after hyperthermia, and a 29% increase in uptake in the small bowel, predominantly in epithelial cells at the tips of villi. This suggests a gradient of metabolic activity and therefore hypoxic stress within villi. Hall et al suggested that heat increases the activity of ATP-dependent membrane pumps and therefore promotes ATP consumption. HSPs uncouple oxidative phosphorylation, thereby slowing ATP production. Hyperthermia promotes glycolysis, generating lactate and ATP. Increasing proton concentration within the cell reduces mitochondrion electron transfer rates and thus oxygen handling by the cell is impaired. The overall picture is one of low intracellular pH, abnormal mitochondrial function and disrupted intracellular ion homeostasis leading to increased oxygen consumption but abnormal oxygen use leaving cells effectively hypoxic and ATP-depleted.

Of interest to this thesis, the HSR can also be induced by the hypothermic treatment of rats (Matz et al, 1996) and mice (Cullen and Sarge, 1997). The interaction between the HSR and hypothermia is an active field of research at present. Other inducers of the HSR include proteasome inhibitors (Pritts et al, 2002), geranylgeranylacetone administration (Ooie et al, 2001) and intraperitoneal or intra-arterial injection (Tsuruma et al, 1999) of sodium arsenite.

HSR and IIR

Whole body hyperthermia has a beneficial effect on intestinal metabolic changes after minimal intestinal handling in rats: heat preconditioning results in prevention of lipid peroxidation, disruption to glucose transport and activation of phospholipase A₂. Induction of heat shock proteins 70 and 90 was looked for, but was not found to have occurred (Prabhu and Balasubramanian, 2002). Pre-conditioning by thermotolerance induces HSP 72 in rats, and when used prior to IIR resulted in less reduction in rolling velocity of leukocytes in mesenteric post-capillary venules, fewer adherent leukocytes and fewer migrated leukocytes compared to animals not pre-treated with thermotolerance (Chen et al, 1997). Whole-body hyperthermia prior to IIR injury in rats increased HSP 70 and heat

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shock factor-1 mRNA expression and resulted in resistance to morphological changes after IIR and attenuated suppression of acetyl-choline-induced secretion. No change was noted to IIR-related decreases in mucosal resistance or sodium-linked glucose absorption. The time-course of HSP induction was demonstrated in this experiment: HSP 70 and heat shock factor-1 mRNA reached a maximum 2 hours after hyperthermia, and declined thereafter; HSP 70 production peaked 4 hours after hyperthermia, and remained high for 12 hours (Fleming et al, 2002). Using a 5-day pre-conditioning programme to upregulate HSP 72 in rats prior to IIR abrogated the reduction in leukocyte rolling velocity in mesenteric vessels, and reduced leukocyte adherence and migration after IIR. Upregulation of HSP 72 was confirmed by Western blot analysis (McCormick et al, 2003). Hyperthermia prior to IIR in rats demonstrated increased HSP 72 production, and a protective effect of hyperthermia on IIR-induced mucosal injury, neutrophil infiltration and leukotriene B₄ production. No effect on increased prostaglandin E₄ production after IIR was noted with prior hyperthermia (Stojadinovic et al, 1995). HSP 73 expression induced by an intra-arterial injection of sodium arsenite prior to IIR resulted in a reduction in the mean peak plasma values of TNF- α and IL-8, a reduction in intestinal tissue MPO levels, and an increase in mean peak plasma IL-10 levels after IIR in rats (Tsuruma et al, 1999). Intraperitoneal sodium arsenite induction of the HSR in mice prior to sepsis resulted in abrogated increases in mucosal permeability and increased plasma levels of IL-10. The beneficial effect of HSR induction on mucosal permeability was abolished by treatment with anti-IL-10 antibodies, implying that IL-10 and the HSR interact in some way to provide protection against sepsis-induced intestinal injury (Wang and Hasselgren, 2002).

HSR and other organs

Prior treatment with hyperthermia before heart donation resulted in increased expression of HSP 72 and improved cardiac graft function after cold storage and transplantation in rats (Gowda et al, 1998). Induction of HSP 72 expression with geranylgeranylacetone prior to cardiac harvest improved

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functional recovery after global ischaemia followed by reperfusion in isolated perfused rat hearts (Ooie et al, 2001). Induction of the HSR was beneficial in lung injury after infra-renal aortic clamping followed by reperfusion in rats: pre-treatment with hyperthermia increased HSP 72 expression in the lungs, intestines and mesentery, and reduced lung oedema and neutrophil infiltration of the lungs after I/R. (Javadpour et al, 1998)

A review article by Yenari (Yenari et al, 1999) discussed the benefits of HSP 70 over-expression via gene transfer and the use of transgenic animals in relation to brain injury of various types.

Conclusion and Objectives

Despite extensive research into the pathophysiological consequences of IIR, mortality and morbidity of IIR remains very high in both adult and paediatric patients. Improved medical, anaesthetic, surgical and intensive care practice may have reduced the incidence, morbidity and mortality of some causes of IIR (acute mesenteric ischaemia, strangulated intestinal hernia, severe sepsis) but the total clinical burden of IIR remains high. The unique anatomy of the intestinal villous and the blood supply to the liver contribute to the impact of IIR on local and remote organs. The ischaemic insult to the intestines initiates an inflammatory cascade that is propagated on intestinal reperfusion by the reintroduction of oxygen and release of reactive oxygen and nitrous oxygen species, the expression of adhesion molecules, the migration and activation of leukocytes and the release of bioactive molecules. This could lead to increased intestinal permeability (possibly followed by the escape of endotoxin and bacteria into the bloodstream), increased vascular permeability both locally and in remote organs, and histological damage to the intestines and distant organs including the liver, lungs and heart. Of interest to clinicians is the changing response to IIR with maturity that may inform therapeutic strategies in different age groups. Novel approaches to

moderating IIR injury include hypothermia and the induction of the HSR, but no specific treatment has yet been found for IIR and its sequelae.

Although research into the consequences of IIR has been ongoing for many years, there are still gaps in our knowledge, especially in the synthesis of different aspects of IIR injury and treatment. The differential impact of IIR at different ages is also only sparsely covered, and the mechanisms of benefit of such strategies as hypothermia have not yet been clearly elucidated.

In this thesis four main hypotheses are addressed. The lack of evidence that mature and immature animals and humans react in the same way to IIR (pages 65 – 67) leads to the **first hypothesis**: young animals will exhibit more severe and possibly a different pattern of hepatic metabolic derangement after IIR than adult animals. The **second hypothesis** is that IIR will induce significant changes in blood composition and splanchnic blood flow. The **third hypothesis** is that IIR-related effects may be abrogated by whole-body hypothermia both locally and systemically. The **fourth hypothesis** is that it may be possible to use near infra-red spectroscopy (NIRS) to follow oxygenation changes in the intestines during IIR – an observation that may improve clinical monitoring of at-risk intestines.

Therefore the aims of this thesis are four-fold. Firstly: to describe the consequences of IIR on the tissue biochemistry of the liver in the suckling rat, and to compare these to those of the adult rat. Secondly: to describe the consequences of IIR in the adult rat on blood composition, hepatic inflow, and cytokine and endotoxin levels in the portal and systemic circulation. Thirdly: to use these studies to assess and analyse whole-body hypothermia as a therapeutic intervention for IIR injury in a rat model. Fourthly: to use NIRS to monitor intestinal tissue oxygenation during IIR in a rat model.

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Chapter 2: Liver metabolites after intestinal ischaemia-reperfusion

Introduction

Intestinal ischaemia-reperfusion (IIR) is associated with injury to several remote organs – for example the liver (Fu et al, 1997), lungs (Wada et al, 2001), heart (Yao et al, 1996) and kidneys (Rothenbach et al, 1997). Remote organ injury (ROI) can lead to the development of multiple organ dysfunction syndrome (MODS) that in turn may progress to multiple organ failure and death. This has been demonstrated in both clinical (Harward et al, 1993; Morecroft et al, 1994) and experimental (Aneman et al, 1996; Poggetti et al, 1992c) settings. The mechanism of induction of ROI after IIR has not been clearly defined, and several groups have demonstrated a delay in, or occasionally complete prevention of, the development of ROI after IIR by a variety of interventions indicating that reactive oxygen species (Poggetti et al, 1992c), activated leukocytes (Horie et al, 1996; Poggetti et al, 1992b), pro-inflammatory bioactive molecules (Souza et al, 2000) and release of endotoxin (Harkin et al, 2001) may be involved in the induction of ROI after IIR. Liver dysfunction after IIR is an early feature in both animals (Horie et al, 1998) and patients (Morecroft et al, 1994), and appears to require intestinal reperfusion (Nakamura et al, 2001; Veichapipat et al, 2001) for a sustained period before hepatic injury is seen (Turnage et al, 1996), although reduction in bile flow (an ATP-dependent, energy consuming process) is seen very early in intestinal reperfusion (Nakamura et al, 2001). Experiments have shown that IIR is associated with activation of Kupffer cells by intestinal effluent (Towfigh et al, 2000); reduction in bile flow rate (Poggetti et al, 1992a; Turnage et al, 1994); neutrophil sequestration within the liver (Kubes et al, 2002); increased hepatic capillary permeability (Sun et al, 2002); loss of the ‘hepatic arterial buffer response’

(Nakamura et al, 2001); release of hepatic transaminases into the systemic circulation (Fu et al, 1997; Yamagishi et al, 2002); and histological damage (Savas et al, 2003).

The metabolism of the liver has also been shown to be adversely affected by IIR in several studies: adenosine triphosphate (ATP) in liver tissue extracts fell after IIR (Turnage et al, 1996); adenosine diphosphate (ADP) and inorganic phosphate (Pi) in liver tissue extracts rose after IIR (Changani et al, 1998); and *in vivo* real-time ³¹P magnetic resonance spectroscopy (MRS) of the liver during IIR demonstrated a loss of high energy adenosine nucleotides and a rise in Pi, with a short lag time after the initiation of intestinal reperfusion in rats (Vejchapipat et al, 2001). Hepatic lactate levels rose after IIR (Vejchapipat et al, 2001), and the liver switched from lactate extraction to lactate production at low hepatic flow rates, similar to those seen after IIR (Samsel et al, 1991). Hepatic hypoperfusion of the degree seen after IIR was associated with hepatic insulin resistance, hyperinsulinaemia and hyperglycaemia (Ma et al, 2003). Loss of hepatic transaminases after IIR (Akçakaya et al, 2002) may partially explain the rise in hepatic tissue alanine demonstrated by Vejchapipat et al (Vejchapipat et al, 2001). Hepatic mitochondrial redox status, as reflected by a fall in the ketone body ratio (acetoacetate to 3-hydroxybutyrate), was found to be compromised after prolonged IIR (Poggetti et al, 1992c). A reduction in hepatic oxidative drug metabolism was found after IIR in dogs (Gurley et al, 1997). An increase in oxidised (but not total) glutathione concentration in liver tissue after IIR in rats has been demonstrated (Turnage et al, 1991). It is clear that the metabolic sequelae of IIR in the liver are complex, but may help to shed light on the mechanism by which IIR induces ROI.

All of the studies describing the effect of IIR on hepatic metabolites detailed above were performed on adult animals. There are subtle differences in the pattern and severity of MODS experienced by adult and paediatric patients during severe illness. Severely septic paediatric patients are noted to develop sequential respiratory, hepatic and renal failure (Doughty et al, 1998), and infants with severe

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necrotizing enterocolitis most frequently experience respiratory, renal, cardiovascular and hepatic failure (Morecroft et al, 1994). Adult severely septic patients experience cardiovascular compromise, followed by pulmonary, renal and hepatic impairment (Brun et al, 1995); a complicated post-operative course in surgical patients is also often heralded by cardiovascular compromise first, followed by respiratory and renal insufficiency, then hepatic and haematological failure (Bush et al, 1995; Oshima et al, 2000). It is possible that the impact of IIR on the immature liver may not follow the same pattern (either in terms of extent and/or nature of derangement) as that seen in adults. In part this may be because the metabolic pathways available to the liver continue to mature after birth. Neonatal rat metabolism is geared towards using a lipid dominated diet (breast milk) to generate energy via β -oxidation, changing towards a carbohydrate-based diet after weaning (Girard et al, 1992; Henning, 1981). Neonatal rat livers do appear to have the requisite antioxidant enzymes available to detoxify some IIR generated metabolites (Muniz et al, 2000), so some resistance to the metabolic effects of IIR ought to be present. However, given the overall metabolic milieu, the fate of substrates and toxins delivered to the liver after IIR may vary with the maturity of the patient or animal affected. Neonates might also be expected to have smaller physiological reserves (for example, smaller circulating blood volume and larger surface-area to volume ratio resulting in greater insensible fluid losses and greater impact of such losses) than fit adults to withstand the systemic effects of IIR. Therefore neonates could experience greater hepatic metabolic disturbance for a given intestinal insult than fit adults due to poorer systemic compensation for the insult given.

It is hypothesized that IIR will result in derangement of hepatic metabolites in both suckling and adult rats, and that the pattern of derangement will not be the same in the two age groups.

The aim of this study was to establish the effect of IIR on selected liver metabolites in suckling rats, and to compare the response to that seen in adult rats.

Materials and Methods

Animal preparation

Rats within two age ranges were studied. Suckling rats were mixed sex Sprague-Dawley of 11 – 13 days old, weighing between 16 and 32g [median weight was 22g]. Young adult rats were age-matched male Sprague-Dawley of 8 to 12 weeks old, weighing between 240 - 310g [median weight was 279g]. Suckling rats were kept with their dams and allowed to suckle freely until anaesthetised. The family group was kept under standardised conditions for light and temperature. Dams were kept under standardised conditions for food and water and allowed access to standard rat chow and water *ad libitum*. Young adult rats were kept under standardised conditions for food, water, light and temperature, and had access to standard rat chow and water *ad libitum*.

An initial insult of 45 minutes of intestinal ischaemia and 60 minutes of intestinal reperfusion was tolerated well by the suckling rats, so the experiment was extended to use an insult known to induce liver metabolite derangement in adult rats (90 minutes of intestinal ischaemia and 60 minutes of reperfusion).

Animal numbers reported below refer to animals completing the entire experimental protocol.

There were nine experimental groups:

- A) Suckling rats, sham operation for 105 minutes (n=18)
- B) Suckling rats, intestinal ischaemia only for 105 minutes (n=18)
- C) Suckling rats, intestinal ischaemia-reperfusion (45 minutes ischaemia + 60 minutes reperfusion) (n=18)
- D) Suckling rats, sham operation for 150 minutes (n=18)
- E) Suckling rats, intestinal ischaemia only for 150 minutes (n=18)
- F) Suckling rats, intestinal ischaemia-reperfusion (90 minutes ischaemia + 60 minutes reperfusion) (n=18)
- G) Adult rats, sham operation for 150 minutes (n=6)

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- H) Adult rats, intestinal ischaemia only for 150 minutes (n=7)
- I) Adult rats, intestinal ischaemia-reperfusion (90 minutes ischaemia + 60 minutes reperfusion) (n=7)

Rectal temperature was monitored continuously immediately after induction of deep surgical anaesthesia, and maintained at $37 \pm 0.5^{\circ}\text{C}$ using a heating pad and radiant lamp.

Surgical procedure

All animals were anaesthetised with 3% halothane by inhalation in a 50:50 mixture of oxygen and nitrous oxide at a flow rate of 200ml/min per gas, until deep surgical anaesthesia had been induced. Anaesthesia was maintained using 0.8 – 1.5% halothane in a 50:50 mixture of oxygen and nitrous oxide at the same flow rates for the remainder of the experiment. Suckling animals had a transverse laparotomy and adult animals had a longitudinal laparotomy. All animals underwent exposure and dissection of the superior mesenteric artery (SMA). Groups A, D and G (sham operation) had no further surgical intervention: the abdominal contents were returned to the abdominal cavity, moistened with warmed normal saline, the abdominal wall approximated and the abdomen covered with a damp swab for the remainder of the experiment (105, 150 and 150 minutes respectively). All other groups (intestinal ischaemia only [IO] and IIR) underwent occlusion of the SMA by a small atraumatic sprung clip. Cessation of pulsation in the arteries of the small bowel mesentery was confirmed visually. The abdominal contents were returned to the abdominal cavity, moistened with warmed normal saline, the abdominal wall was approximated and the abdomen covered with a damp swab. Groups B, E and H had no further surgical intervention and intestinal ischaemia was maintained for the remainder of the experiment (105, 150 and 150 minutes respectively). Groups C, F and I underwent removal of the clip after a period of intestinal ischaemia (45, 90 and 90 minutes respectively). Reperfusion of the mid-gut was confirmed by direct observation of the return of pulsation in the arteries of the small bowel mesentery. The abdominal contents were returned to the abdominal cavity, moistened, and the abdomen was covered again. Reperfusion was permitted without further intervention for 60 minutes in all groups.

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At the end of the experiment, all groups had the right lobe of the liver resected rapidly and immersed in liquid nitrogen. In all cases the liver sample was immersed in liquid nitrogen within 5 seconds of the start of resection.

Tissue extraction and metabolite measurement

Liver tissue was stored in aluminium foil at -80°C until processed (median length of storage was 8 days, range 1 – 50 days). Each suckling liver yielded approximately 0.5g of tissue. In order to achieve a sufficiently high concentration of hepatic metabolites for ^{31}P MRS, three suckling livers were pooled to provide a single sample for metabolite extraction of approximately 1.5g. Adult liver samples were extracted individually. Liver samples were weighed and ground to a fine powder in a pestle and mortar under liquid nitrogen. All chemicals were obtained from Sigma (Sigma Chemical Co., St Louis, MO, USA) except deuterated chemicals that were obtained from Goss (Goss Scientific Instruments Ltd., Essex, UK). Metabolites were extracted using methanol/chloroform/water. Briefly: 2ml/g wet weight of liver of chloroform, 2ml/g wet weight of liver of methanol and 1ml/g wet weight of liver of distilled water were added to the liver powder in a Teflon® coated tube (Nalgene Oakridge Centrifuge tubes, Nalge (Europe) Ltd., Hereford, UK). Once thawed and homogenised using a tissue chopper, the mixture was centrifuged for 40 minutes at 10000rpm at 4°C. The liquid bi-layer was decanted into separate aqueous and chloroform-based fractions and the extraction repeated. The aqueous fractions were pooled, diluted 1:1 with distilled water and lyophilised for 72 hours and it was these that were analysed.

Samples were prepared for MRS as follows: the sample was dissolved in 1200µl of deuterated water ($^2\text{H}_2\text{O}$) and centrifuged at 13000rpm for 5 minutes to remove particulate debris. The sample was split into two and each half (560 - 580µl) placed in a fresh tube. To one half was added 100µl of 10mM TSP (sodium 3-trimethylsilyl-[2, 2, 3, 3, -d₄]-propionate) to provide an internal reference for concentration and chemical shift at 0 ppm for ^1H MRS. pH was adjusted to $7.0 \pm$

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0.05 (uncorrected for ^2H isotope effects – a ^1H pH meter was used and no correction was made for testing for concentration of ^2H , although any differences due to the altered atomic weight of the protons is not expected to be significant) using NaO^2H and ^2HCl . ^1H MR spectral acquisition was completed within the next 4 hours. To the other half of the sample was added 100 μl of 1M EDTA (ethylenediamine tetra-acetic acid) to chelate any divalent metal ions which could degrade the quality of the ^{31}P MRS, and 100 μl of 35mM MDP (methylenediphosphonic acid) as an internal reference for concentration. ^{31}P MR spectral acquisition was completed within the next 12 hours.

An 11.7 T Varian UnityPlus spectrometer (Varian Associates, Palo Alto, California, USA) was used to acquire ^1H and ^{31}P MR spectra at 25°C under the following conditions:

^1H MR spectroscopy: 256 acquisitions, 45° pulse angle, 6 kHz spectral width, 32k data points, repetition time of 9.06 seconds with pre-saturation of the residual $^1\text{H}_2\text{O}/^1\text{HO}^2\text{H}$ resonance.

^{31}P MR spectroscopy: 2048 acquisitions, 45° pulse angle, 13 kHz spectral width, 16k data points, repetition time of 4.06 seconds with ^1H WALTZ decoupling (standard MRS technique using an extra pulse sequence to suppress the residual water signal across a wide chemical shift with very little power, thus minimising heating of the sample interrogated, reducing thermal currents and improving spectral resolution) applied during acquisition.

Peak assignment was based on published data and quantification was effected by comparing integrated peak areas to the integrated peak area of TSP (for ^1H MR spectra) or MDP (for ^{31}P MR spectra) using VNMR deconvolution software (Varian Associates, Palo Alto, California, USA). The calculated amount of each metabolite present was corrected for the number of protons or phosphorus molecules contributing to the measured peaks. Absolute concentration of each metabolite (in $\mu\text{moles/g}$ wet weight of liver tissue) was calculated using the weight of frozen liver originally extracted. Eight metabolites were measured with

¹H MRS (β -hydroxybutyrate, lactate, succinate, glucose, alanine, glutamate, glutamine and glutathione) and three were measured with ³¹P MRS (ADP, ATP and inorganic phosphate). These metabolites were chosen because they are involved in energy, glucose, fat and nitrogen metabolism, and response to oxidant stress.

Statistical analysis

All data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed after testing for normality of data by analysis of variance with Tukey's multiple comparison post-hoc test to compare group means, unless variance was not equivalent in which case unpaired t-tests with Welch's correction for unequal variance and Bonferroni correction for multiple measurements were performed. All statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California, USA).

Results

Figures 2.1 and 2.2 show high resolution ^1H and ^{31}P MR spectra of liver extracts from an adult rat in group G (sham operation for 150 minutes) with the metabolites quantified in this experiment indicated.

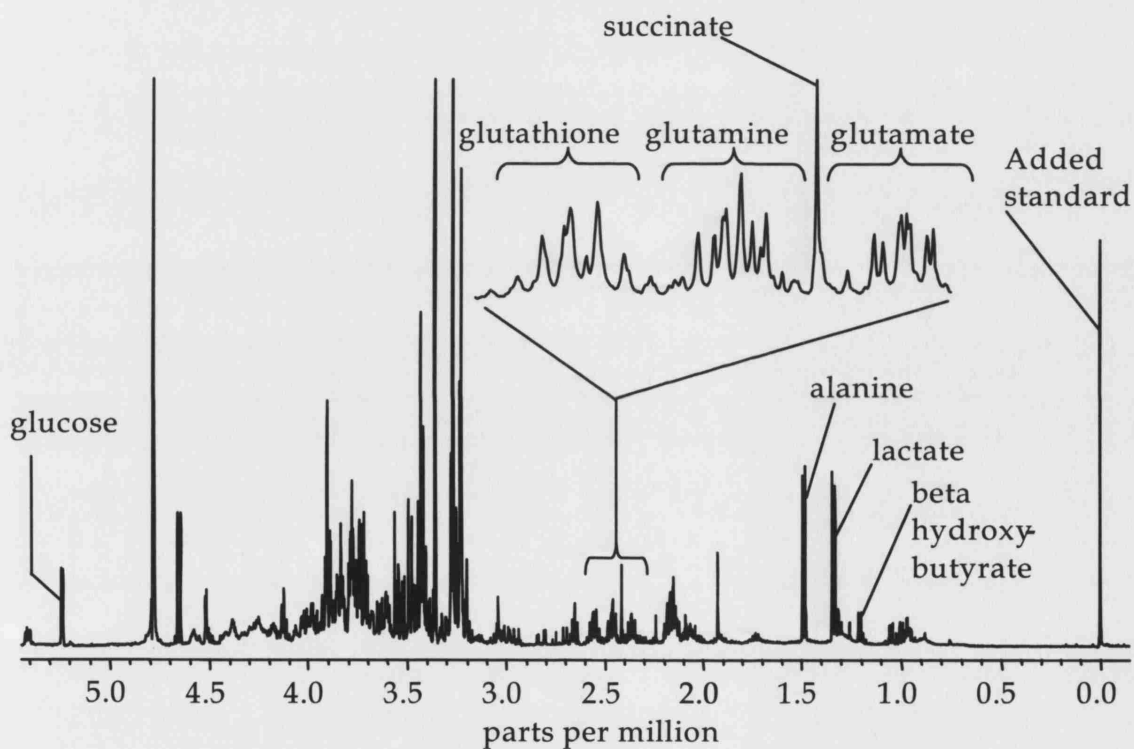


Figure 2.1: High resolution ^1H MR spectrum of liver extract from an adult rat in group G (sham operation for 150 minutes)

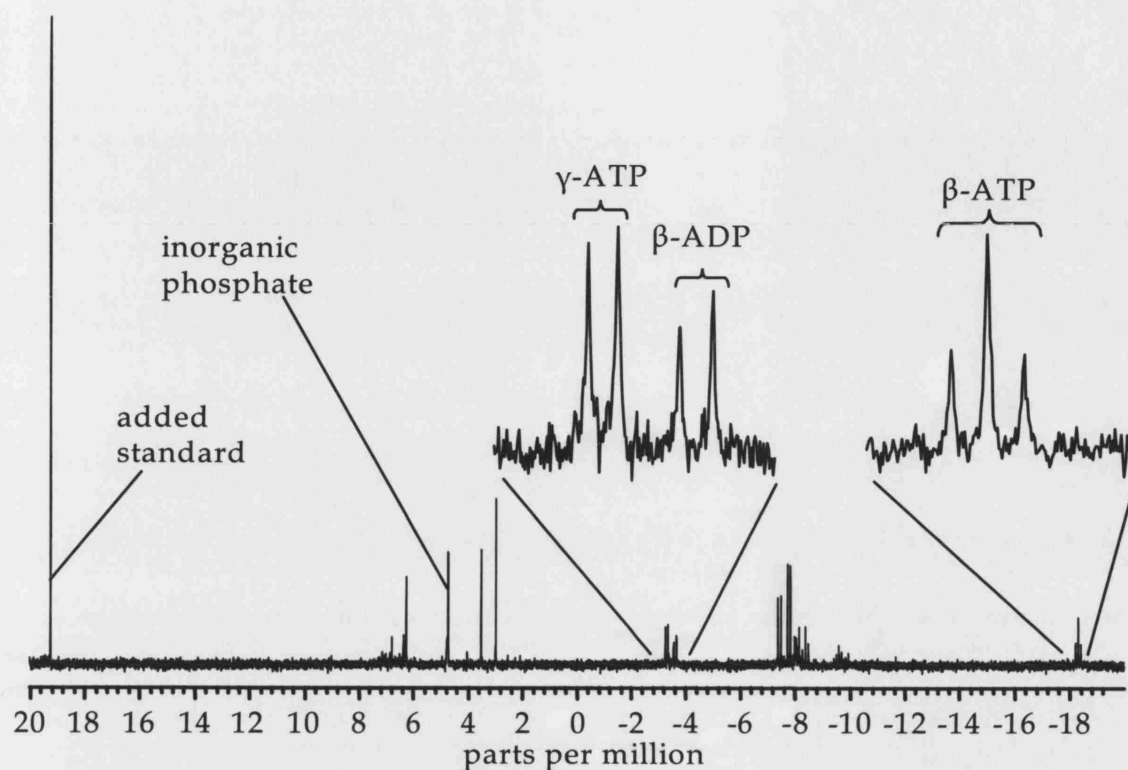
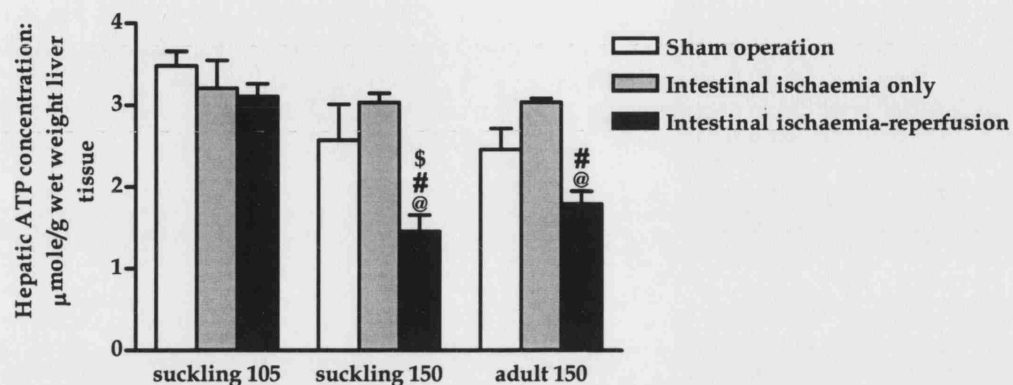


Figure 2.2: High resolution ^{31}P MR spectrum of liver extract from an adult rat in group G (sham operation for 150 minutes)

Metabolites are grouped in high-energy phosphate metabolites (ATP, ADP, Pi); metabolites reflecting gluconeogenesis and ketosis (glucose, succinate, lactate and β -hydroxybutyrate); amino acids (alanine, glutamine and glutamate); and redox status within the liver (glutathione).

Figure 2.3 shows hepatic tissue concentrations of ATP after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.3: Hepatic tissue concentrations of ATP after IIR, IO and sham operation in suckling and adult rats.

$P < 0.05$ versus sham, same age/length of insult*

@ $P < 0.05$ versus intestinal ischaemia only, same age/length of insult

\$ $P < 0.05$ versus suckling 105, same treatment type

*In this set of results only, referring to hepatic ATP, this comparison failed to survive Bonferroni correction for multiple measurements, and must therefore be considered weak evidence at best.

It may be seen from figure 2.3 that in the short suckling group (suckling 105) hepatic ATP concentrations were unaffected by IO or IIR compared to sham operation. In both the long suckling group (suckling 150) and the adult group, hepatic ATP was not altered by IO compared to sham operation, and may fall with IIR compared to sham operation – on initial assessment this comparison yielded a P value less than 0.05, but this failed to survive correction for multiple comparisons and must therefore be considered weak statistical evidence of a difference at best (see legend, figure 2.3). Hepatic ATP was significantly lower after IIR compared to IO in the suckling 150 and adult groups.

Figure 2.4 shows hepatic tissue concentrations of ADP after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.4: Hepatic tissue concentrations of ADP after IIR, IO and sham operation in suckling and adult rats.

@ $P < 0.05$ versus intestinal ischaemia only, same age/length of insult

& $P < 0.05$ versus adult, same treatment type

Figure 2.4 shows that hepatic ADP rose after IO compared to sham operation in both suckling 105 and 150 groups. In the suckling 150 group, ADP was higher after IO than after IIR. No change in hepatic ADP was noted after IO or IIR compared to sham operation in the adult group. Hepatic ADP was higher after IO in the suckling 150 group than in the adult group after the same treatment.

Figure 2.5 shows hepatic tissue concentrations of Pi after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.5: Hepatic tissue concentrations of Pi after IIR, IO and sham operation in suckling and adult rats.

$P < 0.05$ versus sham, same age/length of insult

@ $P < 0.05$ versus intestinal ischaemia only, same age/length of insult

\$ $P < 0.05$ versus suckling 105, same treatment type

& $P < 0.05$ versus adult, same treatment type

Figure 2.5 shows that in the suckling 105 group hepatic Pi did not change significantly after IIR or IO compared to sham operation. Hepatic Pi in the suckling 150 group was lower than in the suckling 105 group after sham operation. In the suckling 150 group, hepatic Pi was significantly higher after IO compared to sham operation, and significantly higher after IIR than after both sham operation and IO. Hepatic Pi levels were higher in the suckling 150 group after IO and IIR than after the same treatment in the adult rat group. Adult rats showed a significant rise in hepatic Pi after IIR compared to sham operation and IO. No change in hepatic Pi was seen after IO compared to sham operation in the adult group.

Figure 2.6 shows hepatic tissue concentrations of glucose after IIR, IO and sham operation in suckling and adult rats.

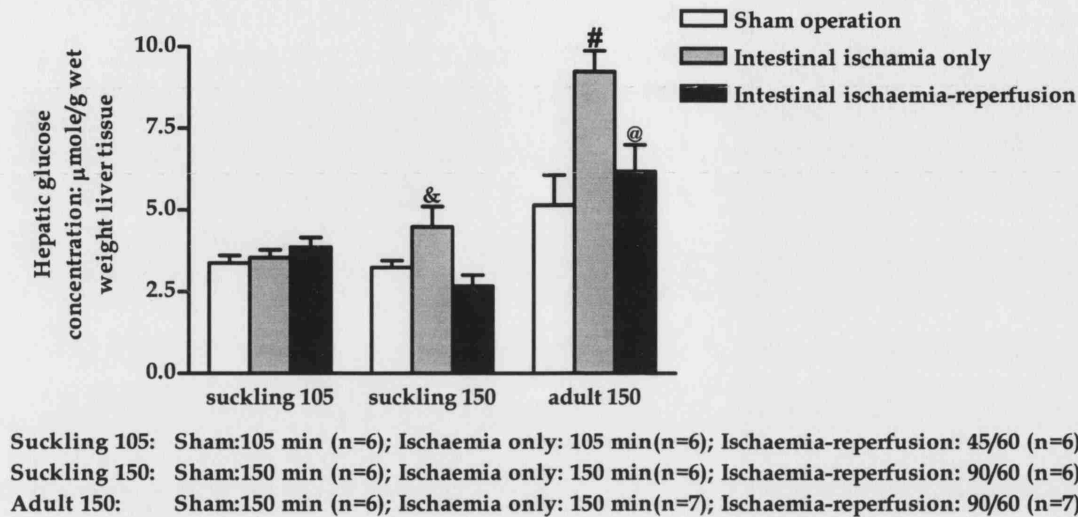


Figure 2.6: Hepatic tissue concentrations of glucose after IIR, IO and sham operation in suckling and adult rats.

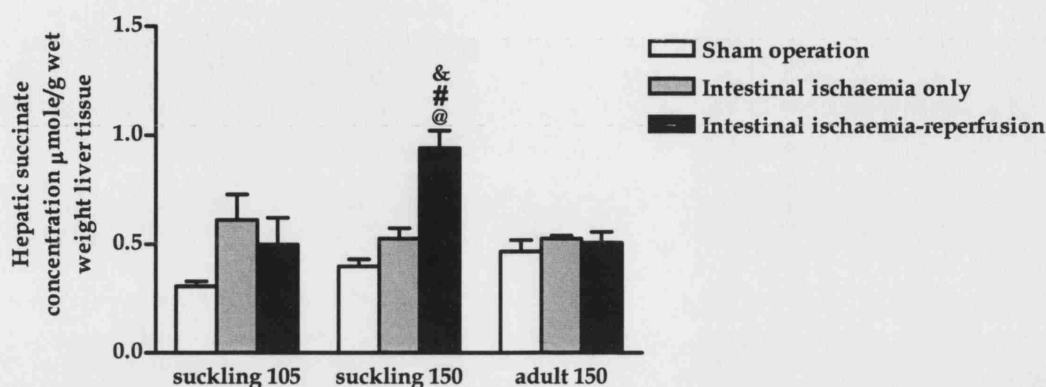
$P < 0.05$ versus sham, same age/length of insult

@ $P < 0.05$ versus intestinal ischaemia only, same age/length of insult

& $P < 0.05$ versus adult, same treatment type

Figure 2.6 shows that hepatic glucose levels did not change significantly after IIR or IO compared to sham operation in either the suckling 105 or 150 groups. After IO, hepatic glucose was significantly lower in the suckling 150 group than in the adult group after the same treatment. In the adult group, hepatic glucose was higher after IO than after sham operation or IIR. No change in hepatic glucose concentration compared to sham operation was seen after IIR in the adult rat group.

Figure 2.7 shows hepatic tissue concentrations of succinate after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.7: Hepatic tissue concentrations of succinate after IIR, IO and sham operation in suckling and adult rats.

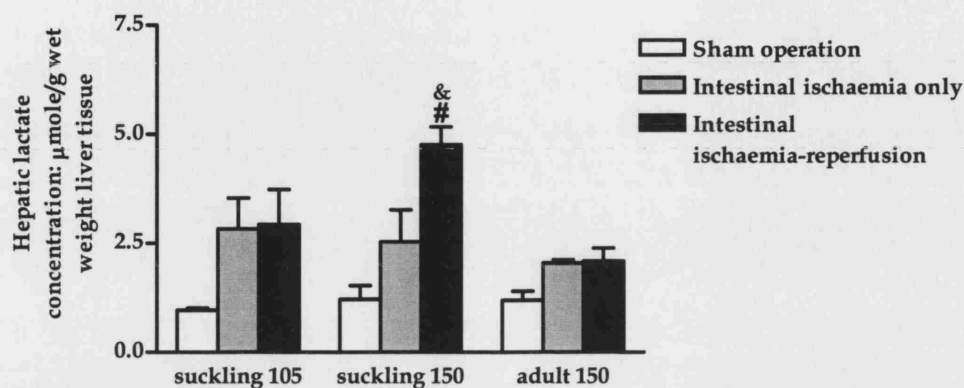
$P < 0.05$ versus sham, same age/length of insult

@ $P < 0.05$ versus intestinal ischaemia only, same age/length of insult

& $P < 0.05$ versus adult, same treatment type

Figure 2.7 shows that hepatic succinate concentration did not change after IIR or IO compared to sham operation in either the suckling 105 or adult groups. In the suckling 150 group, succinate levels were higher after IIR compared to both sham operation and IO. Hepatic succinate levels after IIR were higher in the suckling 150 group than in the adult group after the same treatment.

Figure 2.8 shows hepatic tissue concentrations of lactate after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

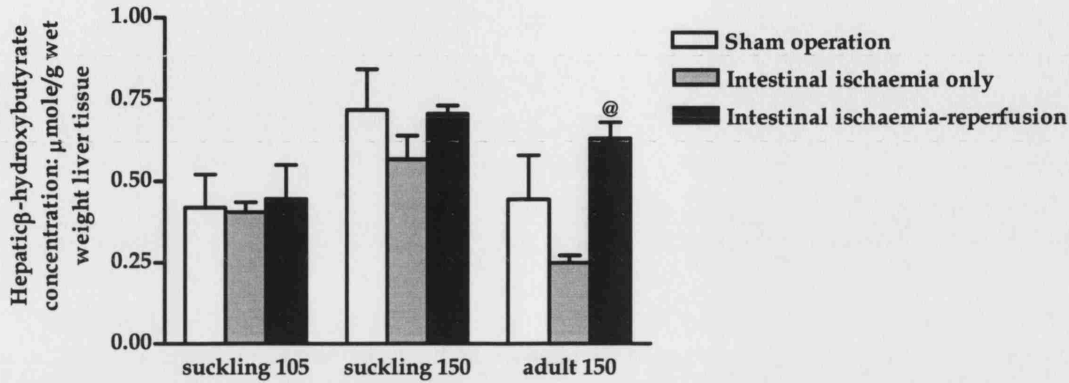
Figure 2.8: Hepatic tissue concentrations of lactate after IIR, IO and sham operation in suckling and adult rats.

$P < 0.05$ versus sham, same age/length of insult

& $P < 0.05$ versus adult, same treatment type

Figure 2.8 shows that hepatic lactate concentration did not change significantly after IIR or IO compared to sham operation in either the suckling 105 or adult groups. In the suckling 150 group, hepatic lactate concentration was higher after IIR than after sham operation, and reached a level significantly greater than that in the adult rat group after the same treatment.

Figure 2.9 shows hepatic tissue concentrations of β -hydroxybutyrate after IIR, IO and sham operation in suckling and adult rats.



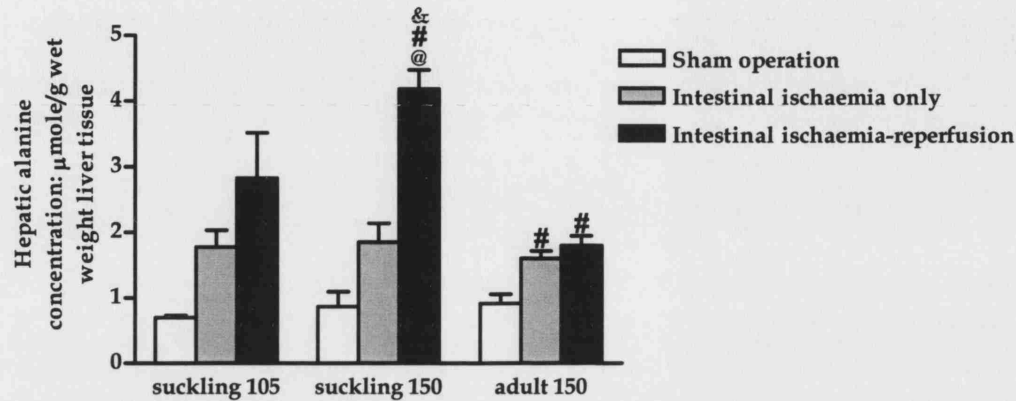
Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.9: Hepatic tissue concentrations of β -hydroxybutyrate after IIR, IO and sham operation in suckling and adult rats.

@ $P < 0.05$ versus intestinal ischaemia only, same experimental group

Figure 2.9 shows that no change in hepatic β -hydroxybutyrate concentration was seen after IIR or IO compared to sham operation in either the suckling 105 or 150 groups. In the adult group, β -hydroxybutyrate was significantly higher after IIR compared to IO.

Figure 2.10 shows hepatic tissue concentrations of alanine after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.10: Hepatic tissue concentrations of alanine after IIR, IO and sham operation in suckling and adult rats.

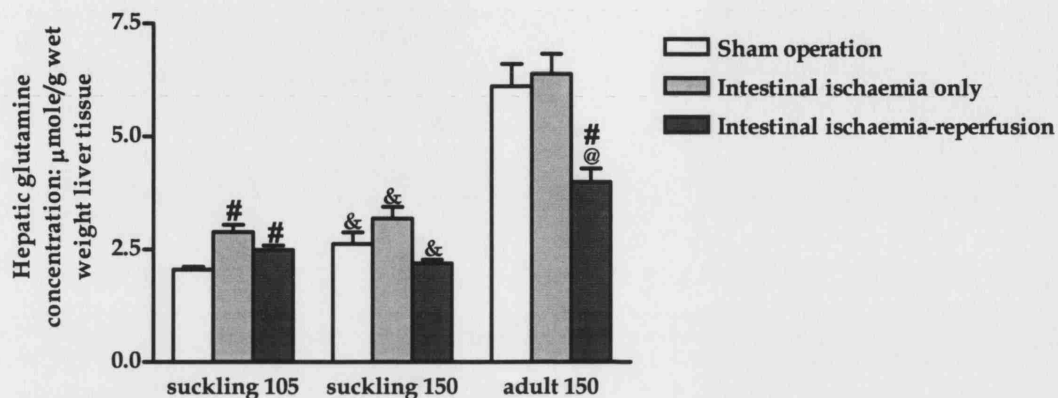
$P < 0.05$ versus sham, same age/length of insult

@ $P < 0.05$ versus intestinal ischaemia only, same age/length of insult

& $P < 0.05$ versus adult, same treatment type

Figure 2.10 shows that hepatic alanine levels did not change significantly after IIR or IO compared to sham operation in the suckling 105 group. In the suckling 150 group, hepatic alanine was higher after IIR than after both sham operation and IO, and reached a level significantly higher than in the adult group after the same treatment. In the adult group, hepatic alanine was higher after IO and IIR compared to sham operation, but no difference between hepatic alanine levels after IO and IIR was found.

Figure 2.11 shows hepatic tissue concentrations of glutamine after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.11: Hepatic tissue concentrations of glutamine after IIR, IO and sham operation in suckling and adult rats.

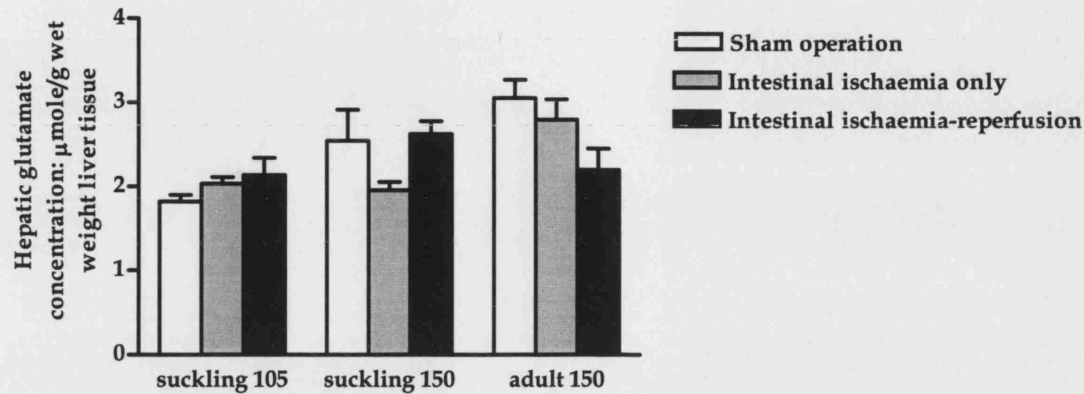
$P < 0.05$ versus sham, same age/length of insult

@ $P < 0.05$ versus intestinal ischaemia only, same age/length of insult

& $P < 0.05$ versus adult, same treatment type

Figure 2.11 shows that hepatic glutamine levels were higher after IO and IIR compared to sham operation in the suckling 105 group. No significant change in hepatic glutamine levels after IIR or IO compared to sham operation was seen in the suckling 150 group, and overall levels were lower in this group for all insults given compared to the adult group after the same treatments. In the adult group, hepatic glutamine levels were lower after IIR than after both sham operation and IO.

Figure 2.12 shows hepatic tissue concentrations of glutamate after IIR, IO and sham operation in suckling and adult rats.



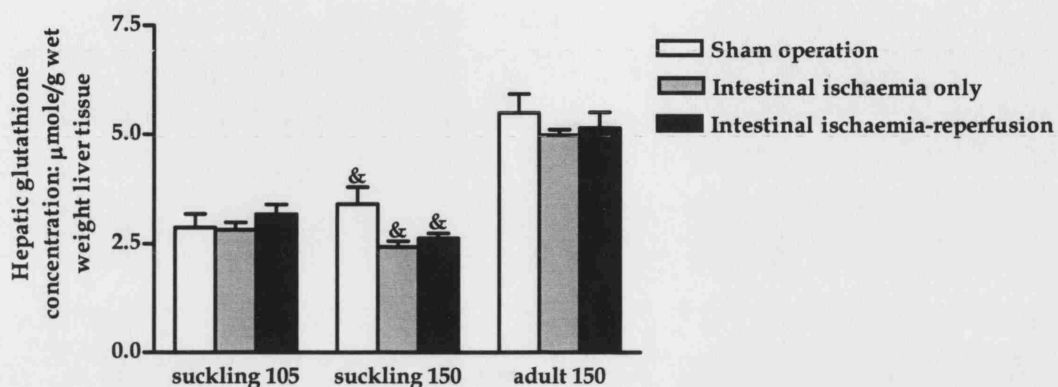
Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.12: Hepatic tissue concentrations of glutamate after IIR, IO and sham operation in suckling and adult rats.

No significant differences with any insult within age/insult length groups, or between any age/insult length groups.

Figure 2.12 shows that no significant differences in hepatic glutamate concentration after IIR or IO compared to sham operation were found within any age/insult length group, or between age/insult groups.

Figure 2.13 shows hepatic tissue concentrations of glutathione after IIR, IO and sham operation in suckling and adult rats.



Suckling 105: Sham:105 min (n=6); Ischaemia only: 105 min(n=6); Ischaemia-reperfusion: 45/60 (n=6)
 Suckling 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=6); Ischaemia-reperfusion: 90/60 (n=6)
 Adult 150: Sham:150 min (n=6); Ischaemia only: 150 min(n=7); Ischaemia-reperfusion: 90/60 (n=7)

Figure 2.13: Hepatic tissue concentrations of glutathione after IIR, IO and sham operation in suckling and adult rats.

& $P < 0.05$ versus adult, same treatment type

Figure 2.13 shows that total hepatic glutathione concentrations did not change significantly after IIR or IO compared to sham operation in either the suckling 105 or 150 groups, or in the adult group. The total hepatic concentration of glutathione was lower in the suckling 150 group than in the adult group for each of the different treatments.

Discussion

Suckling rats given the shorter series of insults (sham operation or IO for 105 minutes, or intestinal ischaemia for 45 minutes followed by intestinal reperfusion for 60 minutes) showed no significant change in hepatic ATP, Pi, glucose, succinate, lactate, β -hydroxybutyrate, alanine, glutamate or glutathione after IIR or IO compared to sham operation. A significant rise in hepatic ADP was seen after IO compared to sham operation; and a significant rise was seen in hepatic glutamine after IO and IIR compared to sham operation. This experimental group served to pilot the techniques required for general anaesthetic and surgery in suckling rats, and tissue-processing with small volumes of hepatic tissue. The general lack of perturbation of the majority of the hepatic metabolites measured after IIR and IO compared to sham operation in this 105 minute group indicates that the intestinal ischaemia +/- reperfusion insults delivered were not substantially more severe than sham operation (that served as the control group) with this suckling animal model. It is noted that the male rat appears to exhibit a more severe response to trauma and haemorrhagic shock than the sexually mature female animal (Caruso et al, 2001; Caruso et al, 2003), and ileal mucous membranes obtained from male rats are more vulnerable to hypoxia \pm acidosis induced barrier failure when tested in Ussing chambers than those obtained from females (Homma et al, 2005). These experiments were performed using sexually mature rats, and it is not clear if such differences pertain in the suckling age group. However, it is noted that male sex is associated with poorer outcome in human neonates in both preterm brain injury (Nunez and McCarthy, 2003) and severe sepsis (Nunez and McCarthy, 2003). These factors make the use of mixed-sex rat pups less than ideal, as confounding factors may have been inadvertently included in this study. It should be noted that failure to use any female rat pups from the litters bought for these experiments would have resulted in their sacrifice which is against stated government policy of 'Replace, Reduce, Refine' for animals used in experiments.

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The sexing of rat pups is not easy, and errors could also have been introduced by the mis-assignment of sex to a particular animal. With the pooling of liver samples this could have increased the numbers of animals used excessively.

In contrast, suckling rats given a longer series of insults (identical to the insults delivered to adult rats– sham operation or IO for 150 minutes, or intestinal ischaemia for 90 minutes followed by intestinal reperfusion for 60 minutes) showed significant changes after IIR in hepatic ATP, Pi, succinate, lactate and alanine compared to sham operation; and after IO in hepatic ADP and Pi compared to sham operation; but no significant changes after either IIR or IO in hepatic levels of glucose, β -hydroxybutyrate, glutamine, glutamate or glutathione.

Adult rats showed significant changes after IIR in hepatic ATP, Pi, alanine and glutamine compared to sham operation; and after IO in hepatic glucose and alanine compared to sham operation; but no significant changes after either IIR or IO in hepatic levels of ADP, succinate, lactate, β -hydroxybutyrate, glutamate or total glutathione compared to sham operation.

The loss of suckling rat hepatic ATP accompanied by a rise in Pi after the longer IIR treatment (significant when compared to IO; significance lost on correction for multiple comparisons when compared to sham operation) closely echoes the findings of other groups examining the effects of IIR on hepatic metabolites using adult animals (Turnage et al, 1996; Vejchapipat et al, 2001). The reason for the drop in hepatic ATP with IIR is not clear – the liver may increase consumption of ATP. This could be caused by production of cytokines in response to inflammatory stimuli arriving from the intestines, or removal of endotoxin released from the intestines and delivered to the liver in the portal vein, or an increased metabolic rate in response to alterations in oxygen delivery and inflammatory stimulation. Alternatively, ATP production may be decreased. This could be due to mitochondrial dysfunction, or reduction in oxygen delivery, or delivered and locally produced toxins preventing efficient electron transfer during oxidative phosphorylation. In either case, it seems that suckling rat liver ATP

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levels are vulnerable to IIR-induced depletion. Pi levels rose with both IO and IIR, and ADP rose with IO, but not with IIR. Taken together, it may be inferred from these results that suckling rats experience some hepatic stress with IO, such that ATP to ADP conversion is increased. This raises hepatic ADP and Pi levels but without the net loss of ATP despite presumed increased conversion to ADP. This may be because ATP production is not compromised with IO, whereas with IIR ATP production is unable to compensate for increased ATP conversion.

Hepatic succinate and lactate both rose after the longer IIR treatment in suckling rats compared to both sham operation and IO, without significant changes in either hepatic glucose or β -hydroxybutyrate. This suggests that there was disruption to the normal functioning of the Krebs cycle in the suckling liver after IIR; but despite this, the liver was able to maintain normal tissue levels of glucose and ketones. This is important as ketogenesis is crucial to the suckling rats to provide fuel for cerebral cells. Succinate and lactate accumulate when glucose is metabolized anaerobically to generate ATP. Some evidence exists in adult animals that hepatic oxygen consumption is not depressed after IIR (Poggetti et al, 1992a), indicating that hepatic hypoxia may not be the root cause of anaerobic glycolysis after IIR. It is possible that hepatic mitochondria are adversely affected by IIR in such a way that oxygen cannot be used, despite no shortage of oxygen supply, thus promoting anaerobic glycolysis. Specific inhibition of the enzymes within the respiratory chain, or the transport molecules moving essential precursors in and out of the mitochondrion could result in this situation. One of the effects of an accumulation of lactate is the generation of an acidic environment that in turn affects the function of regulatory and metabolic enzymes. This effect is well characterized in cardiac muscle (Lee et al, 2003; Scholz and Albus, 1993; Stanley, 2001; Suleymanlar et al, 1992; Zhou et al, 1991).

Hepatic alanine rose steeply with IIR compared to both sham operation and IO in suckling rats with a longer insult, but no changes were noted in the other amino acids measured (glutamine and glutamate). It has been noted in

experiments on adult animals that IIR is associated with a rise in systemic plasma transaminase levels indicating hepatocellular disruption (Akçakaya et al, 2002; Yamagishi et al, 2002; Yamamoto et al, 2001). Loss of transaminases will compromise the liver's ability to use alanine, and hepatocellular disruption will interfere with normal cell metabolism, preventing the usual catabolism of amino acids for gluconeogenesis.

The pattern of change seen adult rats was similar to the changes seen in the suckling rats for ATP (a fall with IIR compared to IO, and possibly sham operation), glutamate and glutathione (no significant differences after IIR or IO compared to sham operation in either age group); a difference in relative changes with the insults given was noted for Pi (increased compared to sham operation with both IO and IIR in suckling rats, but with IIR only in adult rats), alanine (a rise compared to sham operation with IIR only in suckling rats, but a rise compared to sham operation with both IO and IIR in adult rats); and a different pattern entirely for ADP (a rise compared to sham operation with IO in suckling rats, but no significant difference after IIR or IO compared to sham operation in adult rats), glucose (no significant difference after IIR or IO compared to sham operation in suckling rats, but a steep rise with IO compared to both IIR and sham operation in adult rats), succinate and lactate (increased after IIR compared to sham operation in suckling rats, but no significant change after IIR or IO compared to sham operation in adult rats), β -hydroxybutyrate (no significant change after IIR or IO compared to sham operation in suckling rats, but a rise after IIR compared to IO in adult rats) and glutamine (no significant change after IIR or IO compared to sham operation in suckling rats, but a fall with IIR compared to both IO and sham operation in adult rats). Direct comparison of absolute adult and suckling hepatic levels of glutamine and glutathione showed that these metabolites were consistently higher in the adult than in the suckling 150 group after each different treatment.

The reasons behind these age-related differences in hepatic metabolite concentrations after identical treatments may lie in the physiological reserve to withstand the insults administered. This is assumed to be lower in the suckling rat group, as a smaller circulating volume will be more sensitive to intravascular depletion during IIR leading to a greater degree of hypoperfusion late in intestinal reperfusion for a given insult. Alternatively, hepatic enzymes involved in metabolism and detoxification may still be immature in suckling rats. This latter rationale may explain the greater concentration of glutamine and glutathione in adult rat liver, despite the lack of inter-insult changes seen in total hepatic glutathione seen in both age groups. The higher levels of glutamine and the response to IIR seen in adult rats may indicate a preference for glutamine as a gluconeogenic fuel during hepatic stress in the adult animal that is not yet seen in the suckling group. The loss of total hepatic glutamine after IIR could be promoted by the inhibition of glutamine synthetase by raised levels of Pi (Ewart et al, 1995; Watford and Smith, 1990), as found in the adult rat liver after IIR, although it is interesting to note that glutamine synthetase is stimulated by a low pH (Nissim, 1999) as is also found in the liver after IIR. The measurements made in this experiment did not attempt to quantify glutamine flux or consumption, and this may be an interesting field for future work. Perhaps suckling rats do not have access to this metabolic reserve, and so cannot compensate for biochemical stress by using reserves of glutamine. The significance of this is questioned by the lack of perturbation of hepatic glucose levels after IIR.

Hepatic Pi levels after the insults given indicate a greater sensitivity to IO in suckling rats than is seen in adult rats (Pi increased with both IO and IIR in suckling rats, but with IIR only in adult rats). It may be that suckling rats are more sensitive to the drop in hepatic perfusion observed during IO, and therefore experience some increased hepatic Pi production during IO. It should be noted that hepatic ADP also rises in suckling rats with IO – if this ADP is generated from hepatic ATP, this may be the source of the extra Pi seen with IO. The rise in Pi

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levels after IIR seen in both groups of rats is consistent with the results of Vejchapipat et al (Vejchapipat et al, 2001). This may be due to an influx of Pi from the intestine on reperfusion (Taylor et al, 1979) or from the generation of Pi within the liver after IIR (possibly from the dephosphorylation of membrane phospholipids or via cell lysis). It is interesting that the final hepatic concentration of Pi in suckling rats is higher than that of adult rats after IIR – again this suggests a greater sensitivity to the haemodynamic changes seen after IIR, and possibly a smaller capacity to dispose of the extra Pi delivered to the liver after intestinal reperfusion. Conversely, alanine levels in suckling rats did not rise significantly with IO, unlike adult rats, but again reached much higher levels than adult rats after IIR. This demonstrates a metabolite-specific response to the insults delivered. The lack of further rise in hepatic alanine with IIR over IO levels in the adult rat group is at odds with Vejchapipat et al (Vejchapipat et al, 2001). This may be because Vejchapipat et al used an inspired anaesthetic gas mixture of 30:70 (oxygen:nitrous oxide) whereas this experiment used 50:50 (oxygen:nitrous oxide) that might continue to support normal alanine metabolism within the liver.

Hepatic glucose levels did not change significantly with the insults given in the suckling rats, but rose with IO compared to both sham operation and IIR in the adult group. This indicates that the suckling group, although under metabolic stress, and possibly more sensitive to hepatic hypoperfusion during IO as indicated by the rise in Pi seen with IO in this group, are able to maintain normal hepatic glucose levels. Adult rats however, increased hepatic glucose levels with IO, indicating a metabolic stress response to hepatic hypoperfusion. Hepatic glucose levels after IO were twice as high in adult rats, compared to suckling rats. This rise in glucose with IO in adult rats was not accompanied by falls in alanine, glutamate or glutamine – all of which can be gluconeogenic. All animals were fed up until the induction of general anaesthetic, so pre-operative starvation is unlikely to have been a contributory factor. No significant changes after IIR or IO compared to sham operation were seen in β -hydroxybutyrate levels in suckling

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rats, but higher levels were seen after IIR than after IO in adult rats. This latter observation shows an inverse relationship to the rise in hepatic glucose levels in adult rat livers during IO, suggesting a shift away from ketone production during hepatic hypoperfusion, and increased glucose production.

The difference in hepatic succinate and lactate responses to IIR seen between adult and suckling rats may indicate a greater ability of adult rats to continue to use the Krebs cycle under the experimental conditions used in these experiments. The lack of change in adult hepatic succinate and lactate levels after IIR in adult rats is at odds with the work of Vejchapipat (Vejchapipat et al, 2001). This may be because Vejchapipat used an inspired anaesthetic gas mixture of 30:70 (oxygen:nitrous oxide) whereas this experiment used 50:50 (oxygen:nitrous oxide). The extra inspired oxygen may have maintained hepatic tissue oxygenation sufficiently to allow continued aerobic glycolysis. This effect was not seen in suckling rats, which may indicate inability of the immature animal to maintain aerobic glycolysis and/or to dispose of excessive lactate delivered to the liver from the intestines after IIR.

ATP was found to drop substantially compared to IO and possibly sham operation after IIR in adult rats and in suckling rats given a longer insult. The pattern of hepatic responses for this metabolite was very similar for suckling and adult rats given identical insults, despite the differences noted above in the response of other hepatic metabolites. This may indicate a 'final common pathway' of hepatic energy failure after IIR which adult and suckling rats reach by different biochemical pathways. The lack of a drop in hepatic ATP levels in suckling rats given a shorter insult indicates a threshold effect within IIR, below which hepatic energy failure is not induced. This may be related to the duration of intestinal ischaemia, or to the duration of intestinal reperfusion permitted. It is interesting to note that adult rat hepatic ATP was low after IIR despite no change in hepatic lactate levels, possibly indicating a toxic effect of IIR on the liver independent of oxygen delivery and consumption.

Summary and Conclusions

This chapter has described the effect of IIR, IO and sham operation on selected hepatic metabolites measured by ^1H and ^{31}P MRS on suckling and adult rats.

It has been found that a brief insult (105 minutes of sham operation or IO, or 45 minutes of intestinal ischaemia followed by 60 minutes of intestinal reperfusion) results in little perturbation to the metabolite levels in suckling rat livers (a small rise in hepatic glutamine with IIR and IO; a small rise in hepatic ADP with IO). A longer insult (150 minutes of sham operation or IO, or 90 minutes of intestinal ischaemia followed by 60 minutes of intestinal reperfusion) perturbs the hepatic metabolism of both adult and suckling rats, but in subtly different ways. Both age groups show a drop in hepatic ATP with IIR and a rise in hepatic Pi and alanine with IIR. Suckling rats also show a rise in succinate and lactate with IIR, and ADP and Pi with IO. Adult rats show a drop in hepatic glutamine with IIR and a rise in hepatic alanine and glucose with IO. The reasons behind the differences in hepatic responses to the insults given are not clear from this experiment, but age-related responses to hepatic hypoperfusion during IO and IIR, the preference for different substrates for gluconeogenesis and ATP generation when under stress and maturity of enzyme systems within the liver may all be involved. A 'final common pathway' for hepatic failure after IIR is suggested by the similar pattern of ATP depletion seen in both age groups examined.

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Chapter 3: Changes in blood composition after intestinal ischaemia-reperfusion

Introduction

Intestinal ischaemia-reperfusion (IIR) injury alters the composition of blood in both the systemic and portal circulations. Haematocrit rises (Yamamoto et al, 2001), pH falls (Takeyoshi et al, 1999; Yao et al, 1995), inorganic phosphate (Pi) rises (Sawer et al, 1978), enzymes arising from the liver [alanine transaminase (Horie et al, 2002), aspartate transaminase (Fu et al, 1997), alkaline phosphatase] and gut [hexosaminidase (Lobe et al, 1986), β -glucosidase (Morris et al, 1999)] rise, as do lactate dehydrogenase, creatine kinase (Akçakaya et al, 2002), chemokines and cytokines. Intestinal fatty acid binding protein (a marker of intestinal damage) increases in systemic serum after IIR injury (Beuk et al, 2000; Gollin et al, 1993). Endotoxin levels increase in the portal vein (Gathiram et al, 1989), the inferior vena cava (Turnage et al, 1994) and arterial blood (Gaffin et al, 1986). Both increases (Jamieson et al, 1982) and decreases (Altavilla et al, 1996; Souza et al, 2000; Squadrito et al, 1997) in circulating leukocyte numbers after IIR have been described. Red cell deformability is altered making capillary plugging by red cells more likely (Zaets et al, 2003).

Plasma Pi levels have been proposed as a diagnostic aide in the prompt identification and treatment of acute mesenteric ischaemia (Jamieson et al, 1982), but have not been increased consistently pre-operatively in patients later shown to have intestinal ischaemia (May and Berenson, 1983). Experimental work has demonstrated a rise in Pi in systemic and peritoneal fluid after superior mesenteric artery or vein ligation in dogs (Sawer et al, 1978). Urinary Pi also rises after IIR injury, but this was found to be less sensitive than serum or peritoneal Pi as a marker of IIR (Jamieson et al, 1979). Sloughing intestinal mucosa was shown to be

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the source of the increase in systemic serum Pi after IIR injury (Taylor et al, 1979) – Pi therefore had to traverse the liver before it could enter the systemic circulation. The liver would normally assimilate part of the Pi load from the intestines, and any excess would be shed by the kidneys in urine. There is convincing evidence that IIR injury has a deleterious effect on the liver (Nakamura et al, 2001; Turnage et al, 1994), and in this case a large Pi load from the intestines may not be removed effectively. A severely damaged liver may actually contribute to the Pi load entering the systemic circulation as Pi may escape from injured hepatocytes. The biochemical consequences of a raised Pi level in blood delivered to the liver include the inhibition of hepatic enzymes (including glutamine synthetase (Ewart et al, 1995; Watford and Smith, 1990)) and an increase in the local serum anion gap – these effects may enhance IIR-related hepatotoxicity.

Haemoconcentration after IIR injury arises from loss of intra-vascular fluid into interstitial spaces. This is well-described after IIR injury as a combination of epithelial [gut mucosa (Oktar et al, 2002)] and endothelial [vascular endothelium (Ward et al, 2000)] permeability increases that result in the loss of fluid into the intestinal wall (Aydemir et al, 1999) and lumen (Yamamoto et al, 2001) and into the peri-vascular space. Other organs affected by IIR experience endothelial dysfunction and become oedematous, including the liver (Sun et al, 2002), lung (Poggetti et al, 1992) and heart (Yao et al, 1996). Clinically, a raised haematocrit is a non-specific sign of inflammation indicating ‘third spacing’ (loss of intra-vascular fluid into the third space [after arteries and veins, the interstitial space]) and is also seen after severe burns and sepsis. In arterial blood one of the effects of a high haematocrit (in the presence of normal gas-exchange in the lungs) is to increase the amount of oxygen delivered to tissues per millilitre of blood. A high haematocrit is also associated with red cell ‘sludging’ resulting in blocked capillaries, and abnormal flow patterns.

Metabolic acidosis after IIR injury arises partly from the anaerobic use of glucose to produce adenosine triphosphate (ATP), which generates excessive

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lactate that dissociates to form lactate anions and protons. If the bicarbonate buffer system is overwhelmed, blood pH may decrease from the normal range of 7.35 – 7.45 to 7.00 or below. Lactate is removed from the circulation by the liver (in gluconeogenesis by conversion to pyruvate) – if the liver is compromised by IIR injury, lactate removal may be decreased and lactic acidosis could persist. Hydrolysis of ATP also produces a proton, adenosine diphosphate (ADP) and Pi. This contributes to the metabolic acidosis seen after IIR injury, and may help to explain the ongoing acidosis after the reintroduction of oxygen on reperfusion if oxidative phosphorylation cannot recommence due to IIR-induced mitochondrial malfunction. Low pH shifts the sigmoid haemoglobin-oxygen dissociation curve to the right, allowing oxygen to be released more easily within tissues. However, if tissues have been metabolically affected by IIR injury the ability of cells to use delivered oxygen may be compromised, and oxygen consumption could fall despite a local oxygen debt following ischaemia. Evidence indicates that oxygen use during severe sepsis is abnormal. This may be due to malfunction of mitochondria in the presence of reactive oxygen and nitrogen-oxygen species (Singer and Brealey, 1999), and some evidence (Nankervis et al, 2000) indicates that a similar inability to use oxygen may occur after IIR. Therefore, despite high haematocrit and low pH favouring oxygen release to tissues, tissue hypoxia may still be present due to a sub-cellular failure to use available oxygen after IIR injury.

The aim of experiment one (plasma Pi after IIR injury) was to examine the effect of IIR injury on Pi levels in superior mesenteric and right atrial venous blood in a rat model, and to compare superior mesenteric and right atrial venous Pi levels in the same animal during intestinal reperfusion. Sampling times were chosen early in reperfusion (after 2 and 10 minutes of reperfusion), at the mid-point of reperfusion (30 minutes of reperfusion) and at the end of reperfusion (60 minutes of reperfusion) in order to provide a profile of serum Pi levels throughout reperfusion. It was hypothesised that a high Pi load would be found in the

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superior mesenteric vein (SMV) on intestinal reperfusion, and that this would be removed by the liver, resulting in lower Pi levels in the right atrium (RA).

The aim of experiment two (haematocrit, pH and oxygen content after IIR injury) was to document the changes in haematocrit, pH and oxygen content after IIR in our animal model, and to calculate arterio-venous oxygen difference across capillary beds draining into the SMV and inferior vena cava (IVC) after IIR injury. Sampling times were chosen early in reperfusion (after 1 minute of reperfusion) and at the end of reperfusion (after 60 minutes of reperfusion) in order to provide a simple profile of changes in blood composition with reperfusion. It was hypothesised that IIR injury would cause a rise in haematocrit, fall in pH and impair oxygen extraction in the intestines after IIR.

Materials and methods

Experiment 1: Plasma inorganic phosphate after intestinal ischaemia-reperfusion injury

Animal preparation

Age-matched (8 – 12 weeks old) male Sprague-Dawley rats weighing between 210 and 342g (median weight 258g) were studied. Rats were kept under standardised conditions for food, water, light and temperature. All animals had access to standard rat chow and water *ad libitum*. Animal numbers reported below refer to animals completing the entire experimental protocol.

There were six experimental groups:

- A) Sham operation for 100 minutes (n=6)
- B) Sham operation for 150 minutes (n=7)
- C) Intestinal ischaemia-reperfusion (90 minutes ischaemia + 2 minutes reperfusion) (n=6)
- D) Intestinal ischaemia-reperfusion (90 minutes ischaemia + 10 minutes reperfusion) (n=6)

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E) Intestinal ischaemia-reperfusion (90 minutes ischaemia + 30 minutes reperfusion) (n=6)

F) Intestinal ischaemia-reperfusion (90 minutes ischaemia + 60 minutes reperfusion) (n=7)

Rectal temperature was monitored continuously immediately after induction of deep surgical anaesthesia (absence of response to firm web-space pinch), and maintained at $37 \pm 0.5^{\circ}\text{C}$ using a heating pad and radiant lamp.

Surgical procedure

Animals were anaesthetised with 3% halothane by inhalation in a 2:1 mixture of oxygen and nitrous oxide at a flow rate of 1l/min and 500ml/min respectively, until deep surgical anaesthesia had been induced. Anaesthesia was maintained using 0.8-1.5% halothane in a 2:1 mixture of oxygen and nitrous oxide at the same flow rates for the remainder of the experiment. All animals had a silicone catheter inserted into the right external jugular vein (4.2 French gauge, 0.7mm internal diameter single lumen paediatric intravenous catheter, Broviac®, Bard Ltd., Crawley, UK) and advanced into the RA, then flushed with 0.2ml heparinised saline (10 units heparin lithium salt (Sigma Chemical Co, St Louis, MO, USA) per ml)[see figure 3.1, page 145]. Animals had a midline laparotomy and exposure of the superior mesenteric artery (SMA). Groups A and B had no further surgical intervention: the abdominal contents were returned to the abdominal cavity, moistened with warmed normal saline, the abdominal wall approximated and the abdomen covered with a damp swab for a further 100 and 150 minutes respectively. Groups C – F had occlusion of the SMA by a small atraumatic sprung clip. Cessation of pulsation in the arteries of the small bowel mesentery was confirmed by visual inspection. The abdominal contents were returned to the abdominal cavity, moistened with normal saline, the abdominal walls approximated and the abdomen covered with a damp swab for a further 90 minutes. After 90 minutes of intestinal ischaemia, the clip occluding the SMA was

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removed and reperfusion of the midgut confirmed by direct observation of the return of pulsation in the arteries of the small bowel mesentery. Reperfusion was permitted for 2 minutes (Group C), 10 minutes (Group D), 30 minutes (Group E) or 60 minutes (Group F).

1ml blood samples were taken from the SMV by direct puncture, and RA via the internal jugular catheter (see figure 3.1 below), using sterile equipment (21G Microlance®3 needle and 1ml Plastipak® syringe, both from Becton, Dickinson UK Ltd, Cowley, Oxford, UK). Each sample was placed in a 2.0ml capped plastic vial (Safe-Lock, Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany) in which 100µl of heparinised normal saline (8.5 units lithium heparin salt (Sigma Chemical Co, St Louis, MO, USA) per ml) had been evaporated to dryness. Blood samples were centrifuged at 13000rpm for 5 minutes within 2 minutes of withdrawal, and the plasma decanted into a clean capped plastic vial.

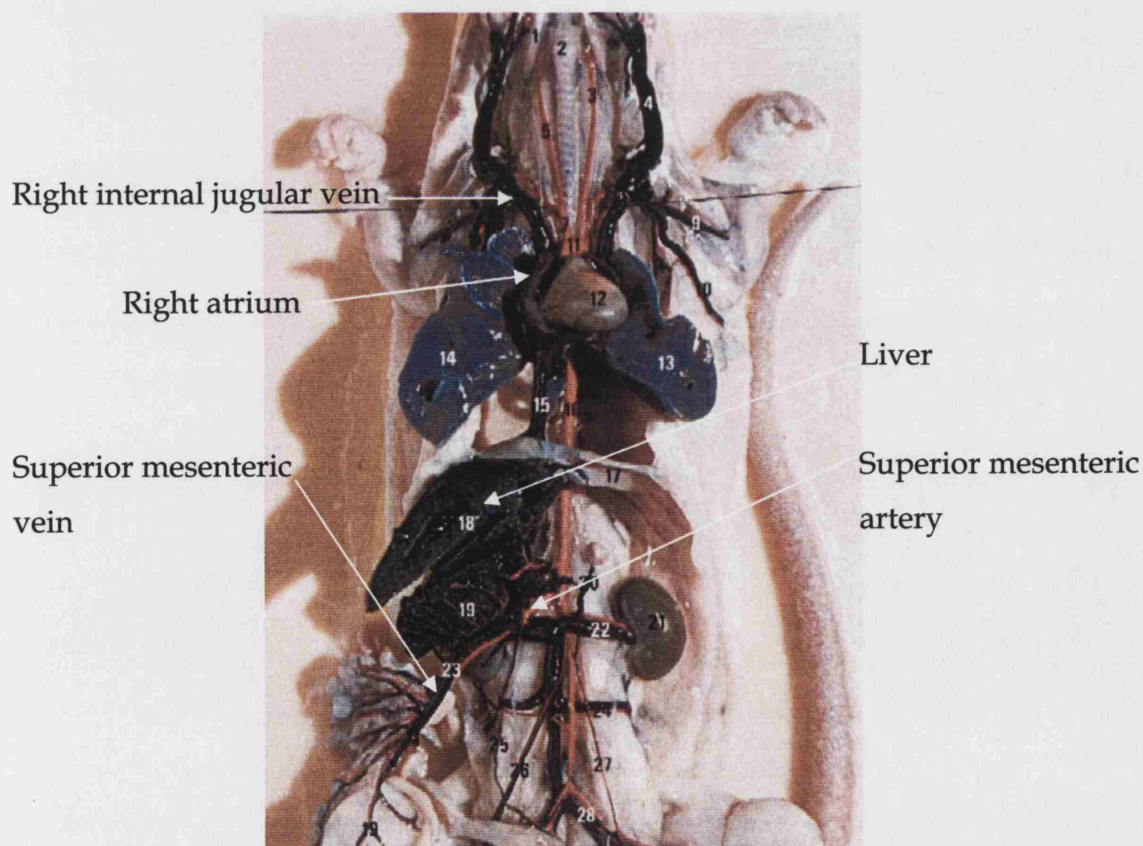


Figure 3.1: Illustration of the sites cannulated, occluded and sampled from in experiment 1.

Measurement of inorganic phosphate

Samples were stored at -20°C until they were processed (median length of storage was 14 days, range 2 – 62 days). Prior to dry-slide analysis for Pi, samples were tested for haemoglobin to assess Pi contamination by haemolysis during sample acquisition and processing. A haemoglobin testing kit was used (Total Hemoglobin, Sigma Diagnostics®, St Louis, MO, USA) to estimate haemoglobin content. Briefly: a standard curve was prepared using change in absorbance at 540nm in solutions containing 0 – 10g/dl of haemoglobin in Drabkin's Solution. 20µl of each plasma sample was added to 1ml of Drabkin's Solution and change in absorbance at 540nm measured against pure Drabkin's Solution. Change in absorbance at 540nm was converted into haemoglobin concentration using the standard curve. The lower limit of detection of the test used was 2g/dl - only one sample was found to be likely to be contaminated with haemoglobin and was excluded from further analysis.

Sample Pi was measured in a hospital biochemistry laboratory (Camelia Botnar Laboratories, Great Ormond Street Hospital for Children, London, UK) using a Kodak® dry-slide analyser (Kodak, USA). Pi levels were recorded by computer. Samples with Pi levels measuring in excess of the quoted accurate range of the analyser were measured again after dilution in standard plasma of known Pi concentration, and original Pi levels calculated manually.

Statistical analysis

All data are presented as mean \pm SEM. Statistical analysis was performed after testing for normality of data by analysis of variance with Tukey's multiple comparison post-tests, or unpaired t-tests with Welch's correction for unequal variance and correction for multiple comparisons (dependent upon equivalence of variance). *P* values of < 0.05 were considered statistically significant. All statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 302 for Windows, GraphPad Software, San Diego, CA, USA).

**Experiment 2: Haematocrit, pH and oxygen content in the portal and systemic circulations
after intestinal ischaemia-reperfusion injury**

Animal preparation

Age-matched (8 – 12 weeks old) Sprague-Dawley rats weighing between 238 and 310g (median weight was 275g) were studied. Rats were kept under standardised conditions for food, water, light and temperature. All animals had access to standard rat chow and water *ad libitum*. Animal numbers reported below refer to animals completing the entire experimental protocol.

There were four experimental groups:

- 1) Sham operation for 91 minutes (n=6)
- 2) Sham operation for 150 minutes (n=5)
- 3) Intestinal ischaemia-reperfusion (90 minutes ischaemia + 1 minute reperfusion) (n=6)
- 4) Intestinal ischaemia-reperfusion (90 minutes ischaemia + 60 minutes reperfusion) (n=6)

Rectal temperature was monitored continuously immediately after induction of deep surgical anaesthesia (absence of response to firm web-space pinch), and maintained at $37 \pm 0.5^{\circ}\text{C}$ using a heating pad and radiant lamp.

Surgical procedure

All animals were anaesthetised with 3% halothane by inhalation in a 50:50 mixture of oxygen and nitrous oxide at a flow rate of 200ml/min per gas, until deep surgical anaesthesia had been induced. Anaesthesia was maintained using 0.8-1.5% halothane in a 50:50 mixture of oxygen and nitrous oxide at the same flow rates for the remainder of the experiment. All animals had a midline laparotomy and exposure of the SMA. Groups 1 and 2 (sham operation for 91 and 150 minutes respectively) had no further surgical intervention. The abdominal contents were returned to the abdominal cavity, moistened with warmed normal saline, the

abdominal wall approximated and the abdomen covered with a damp swab for the remainder of the experiment. Groups 3 and 4 (IIR) underwent occlusion of the SMA by a small atraumatic sprung clip. Cessation of pulsation of the arteries in the small bowel mesentery was confirmed by direct observation. The abdominal contents were returned to the abdominal cavity, moistened with warmed normal saline, the abdominal wall approximated and the abdomen covered with a damp swab. Intestinal ischaemia was allowed for 90 minutes. In Group 3 only a single 0.5ml blood sample was taken from a side branch of the SMV immediately prior to de-occlusion of the SMA (see below for blood sampling details). The SMV side branch was occluded with a haemostat to prevent blood loss taking care not to obstruct flow in the main blood vessel. The SMA was de-occluded by removal of the clip and reperfusion confirmed by direct observation of the return of pulsatile flow in the arteries of the small bowel mesentery. Reperfusion was permitted for 1 minute in Group 3 and 60 minutes in Group 4.

At the end of the experiment 0.5ml blood samples were taken from the SMV, IVC and aorta (see figure 3.2, page 150) into 1ml Plastipak® sterile syringes using 21G Microlance®3 sterile needles (needles and syringes from Becton Dickinson UK Ltd, Cowley, Oxford, UK). Needles and syringes were flushed with heparinised normal saline (25 units lithium heparin salt (Sigma Chemical Co, St Louis, MO, USA) per ml) immediately prior to use.

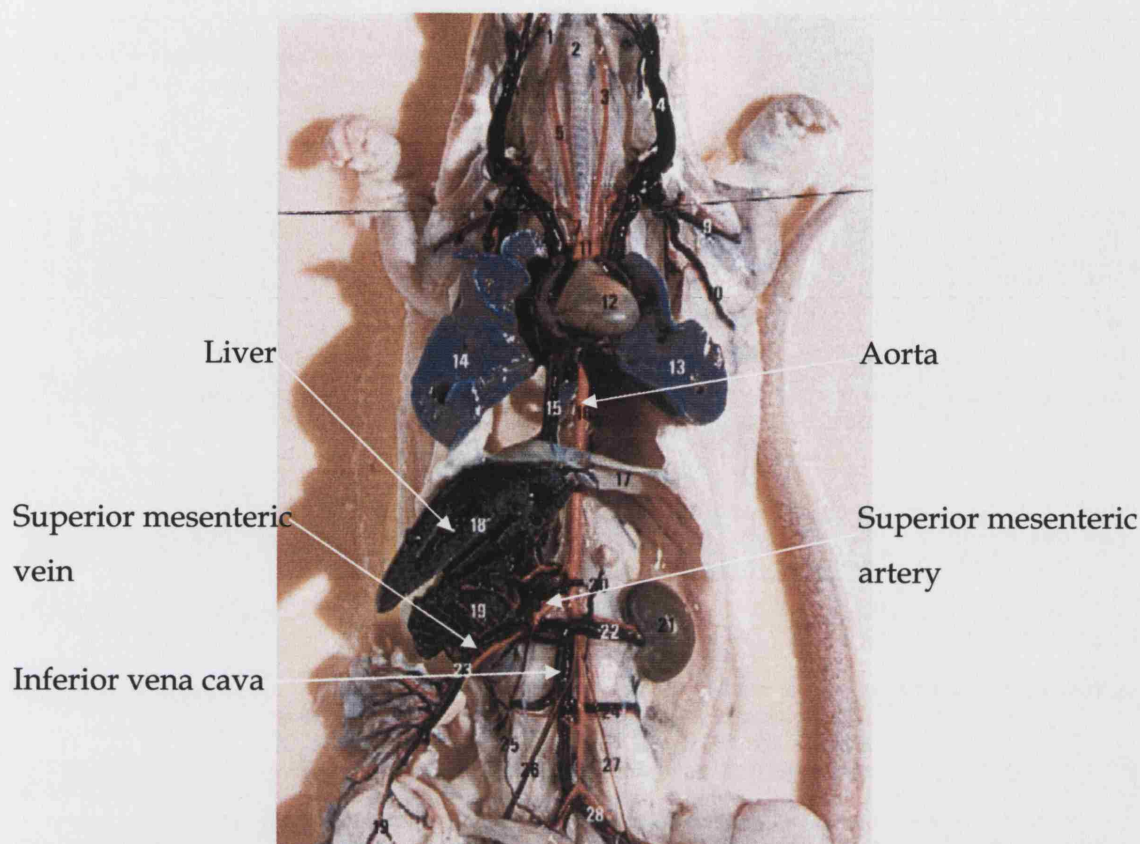


Figure 3.2: Illustration of the blood vessels occluded or sampled from in experiment 2

Measurement of blood gases

All blood samples were used immediately to fill the sample chamber of an EG7+ iSTAT® portable clinical analyser cartridge (iSTAT® Corporation, Princeton, NJ, USA), and analysed straight away using an iSTAT® portable clinical analyser (iSTAT® Corporation, Princeton, NJ, USA). The delay between initiation of sample collection and analysis was less than 2 minutes in all cases. Haematocrit, partial pressure of oxygen (pO_2) and pH were measured directly by the analyser. Haemoglobin concentration and oxygen saturation were calculated automatically by the analyser. Blood oxygen content was calculated manually using the following formula (Dugas et al, 2000) adapted for pO_2 expressed in kPa:

$$C_xO_2 = (1.34 \times Hb \times SaO_2) + (0.0233 \times pO_2)$$

where: C_xO_2 denotes blood oxygen content in vessel 'x' in ml O_2 /dl blood

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Hb denotes haemoglobin concentration in g/dl

SaO₂ denotes haemoglobin oxygen saturation as a percentage

pO₂ denotes partial pressure of oxygen in kPa.

1.34 is a scaling factor indicating the maximum volume of oxygen carried in ml per gram of haemoglobin

0.0233 is the solubility coefficient of oxygen when partial pressure of oxygen is measured in kPa.

All measurements were made at room temperature (20°C – experiments performed in a climate-controlled MRS laboratory with continuous temperature monitoring). It is accepted that oxygen measurements in molar terms would have avoided the need for correction for temperature but this was not possible with the equipment available.

Arterio-venous (A-V) whole blood oxygen difference for the territories drained by the SMV and IVC was estimated by calculating the amount of oxygen extracted per gram of haemoglobin leaving the territory via either via the IVC or SMV, and then converting this figure back into ml O₂/ml blood at the haematocrit of the draining vessel. The starting oxygen content was determined by the whole blood oxygen content in the aorta. The correction for haematocrit was necessary because haematocrit increased differentially during the passage through the two territories examined, and would otherwise have skewed the calculation of A-V oxygen difference.

Statistical analysis

All data are presented as mean \pm SEM. Statistical analysis was performed after testing for normality of data by analysis of variance with Tukey's multiple comparison post-tests, or paired and unpaired t-tests with Welch's correction for unequal variance and correction for multiple comparisons dependent on equivalence of variance. Patterns of behaviour after different treatments across sample sites were compared using 2-way ANOVA. *P* values of < 0.05 after

correction for multiple comparisons were accepted as statistically significant. All statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, CA, USA).

Results

Experiment 1: Plasma inorganic phosphate after intestinal ischaemia-reperfusion injury

Plasma Pi concentration was plotted against plasma haemoglobin concentration for each animal (figure 3.3).

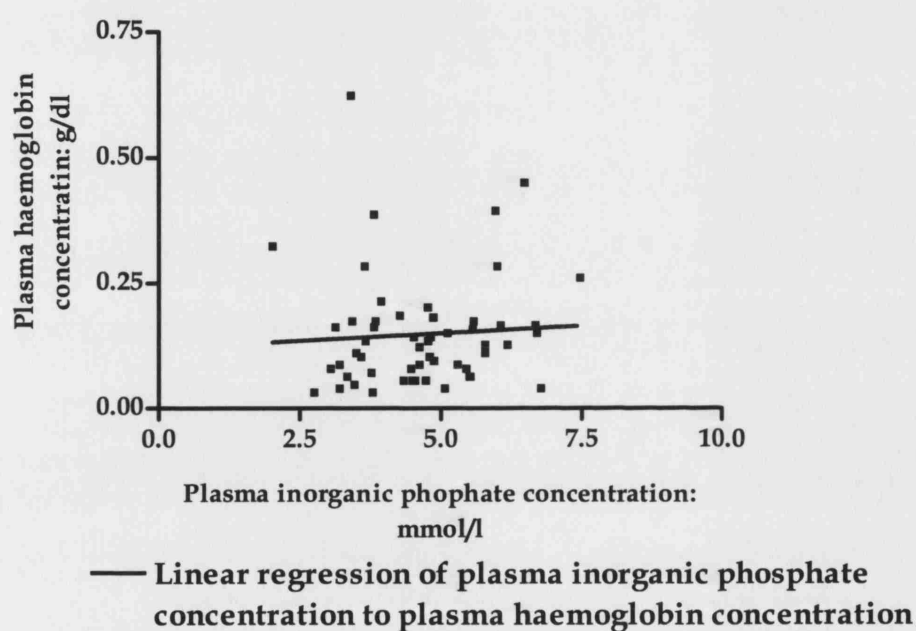


Figure 3.3: Plasma Pi concentration plotted against same sample plasma haemoglobin concentration for all animals in experiment 1.

Linear regression of plasma Pi concentration to plasma haemoglobin concentration: P (slope \neq zero) = 0.6531 - no relationship found.

Figure 3.3 did not demonstrate a significant relationship between plasma Pi and plasma haemoglobin concentrations. This indicates that it is unlikely that red cell lysis contributed to plasma Pi concentrations measured in this experiment.

Plasma Pi concentration in the SMV was plotted against experimental time (figure 3.4).

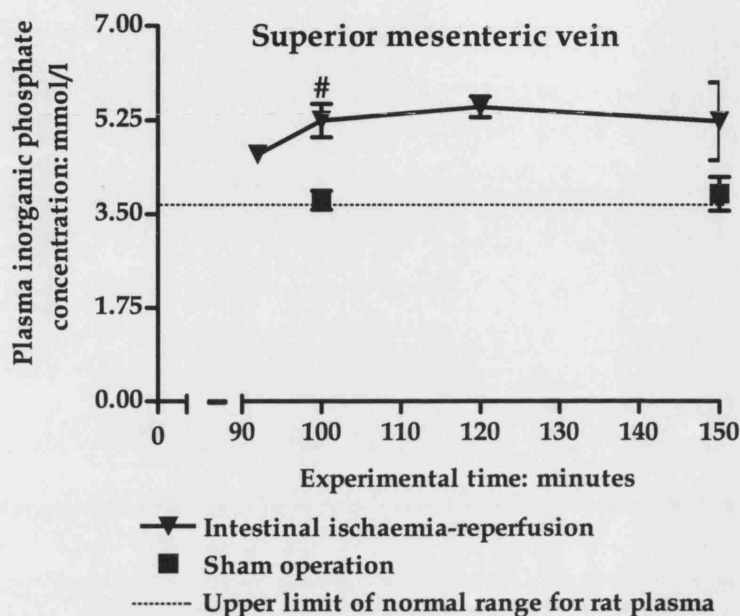


Figure 3.4: Plasma Pi concentration in the SMV after IIR or sham operation.

= $P < 0.05$ versus sham operation at same experimental time.

Figure 3.4 shows that SMV plasma Pi was significantly greater after 10 minutes of reperfusion than after sham operation for 100 minutes, but no progressive rise was seen with increasing reperfusion time (SMV plasma Pi levels after 2 and 60 minutes of reperfusion were equivalent). No change in SMV plasma Pi level was noted with prolonged sham operation.

Plasma Pi concentration in the RA was plotted against experimental time (figure 3.5).

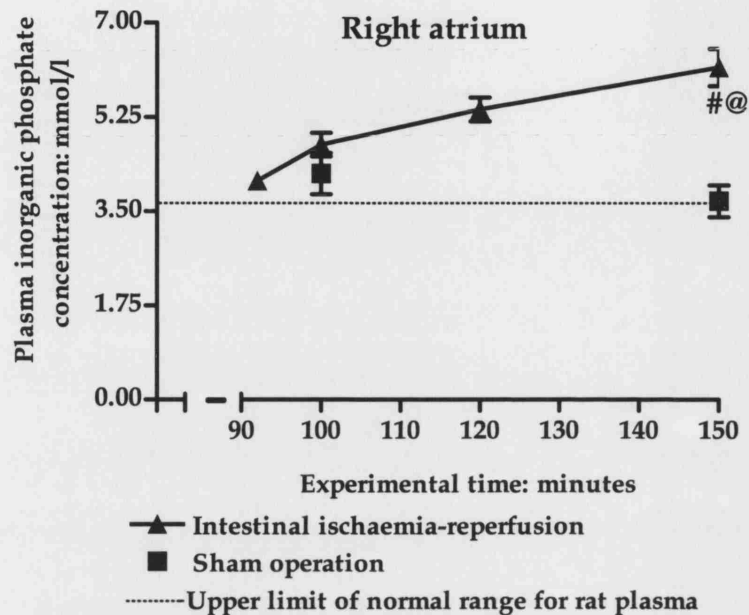


Figure 3.5: Plasma Pi concentration in the RA after IIR or sham operation.

= $P < 0.05$ versus sham operation at same experimental time.

@ = $P < 0.05$ versus intestinal ischaemia followed by 2 minutes of reperfusion

Figure 3.5 shows that RA plasma Pi rose progressively during reperfusion (higher levels were seen after 60 minutes than after 2 minutes of reperfusion). Significantly higher RA plasma Pi levels were seen after 60 minutes of reperfusion than were seen after 150 minutes of sham operation. No change in RA plasma Pi level was noted with prolonged sham operation.

Porto-systemic difference (SMV - RA) in plasma Pi concentration was plotted against experimental time (figure 3.6).

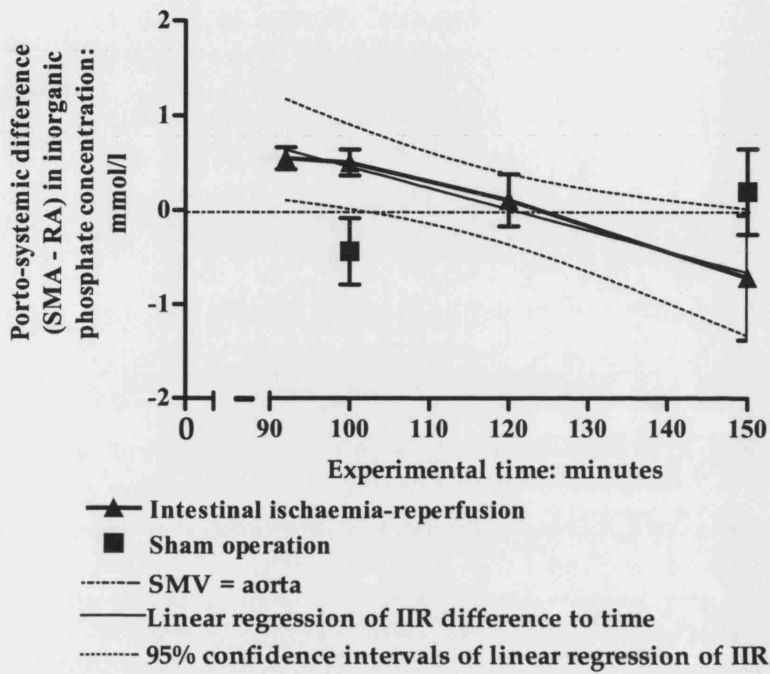


Figure 3.6: Porto-systemic difference (SMV - RA) in plasma Pi levels after IIR or sham operation.

Linear regression of porto-systemic difference in plasma Pi levels after IIR with reperfusion time: $P = 0.0097$ - a significant decline in porto-systemic difference in Pi over time.

Figure 3.6 shows that there was a significant decline in porto-systemic plasma Pi difference with increasing reperfusion time after intestinal ischaemia. No significant change in plasma Pi porto-systemic difference was seen after prolonged sham operation.

Experiment 2: Haematocrit, pH and oxygen carriage in the portal and systemic circulations after intestinal ischaemia-reperfusion injury

Haematocrit measurements from the SMV, aorta and IVC after IIR, intestinal ischaemia only (IO) (in the SMV only) or sham operation are shown in figure 3.7.

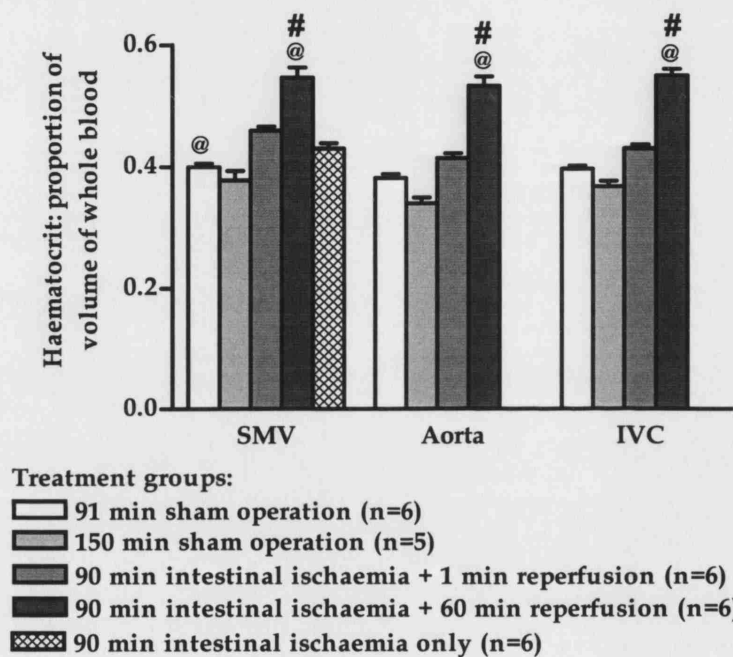


Figure 3.7: Haematocrit after IIR, IO or sham operation in the SMV, aorta and IVC.

@ $P < 0.05$ versus 90 min intestinal ischaemia + 1 min reperfusion for the same sample site

$P < 0.05$ versus 150 min sham operation for the same sample site

Interaction between treatment group and sample site: $P = 0.5777$

Figure 3.7 shows that haematocrit rises after intestinal ischaemia followed by 60 minutes of reperfusion compared to both 150 minutes of sham operation and to 1 minute of reperfusion at all sites. In the SMV only, haematocrit increased with intestinal reperfusion of 1 minute compared to 91 minutes of sham operation. The pattern of change in haematocrit after each treatment was the same at all sites.

pH measurements from the SMV, aorta and IVC after IIR, IO (in the SMV only) or sham operation are shown in figure 3.8.

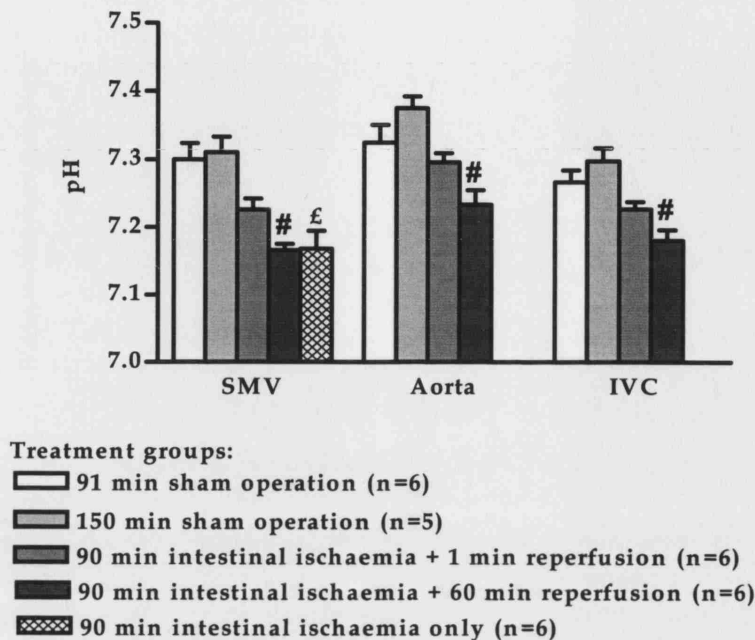


Figure 3.8: pH after IIR, IO or sham operation in the SMV, aorta and IVC.

£ $P < 0.05$ versus 91 min sham operation for the same sample site

$P < 0.05$ versus 150 min sham operation for the same sample site

Interaction between treatment group and sample site: $P = 0.8036$

Figure 3.8 shows that pH falls after intestinal ischaemia and 60 minutes of reperfusion compared to 150 minutes of sham operation at all sites. A sample was taken before reperfusion from the SMV only (i.e. after 90 minutes of intestinal ischaemia but before intestinal reperfusion) and this demonstrated a drop in pH compared to 91 minutes of sham operation. The pattern of change in pH after each treatment was the same at all sites.

Whole blood oxygen content measurements from the SMV, aorta and IVC after IIR, IO (in the SMV only) or sham operation are shown in figure 3.9.

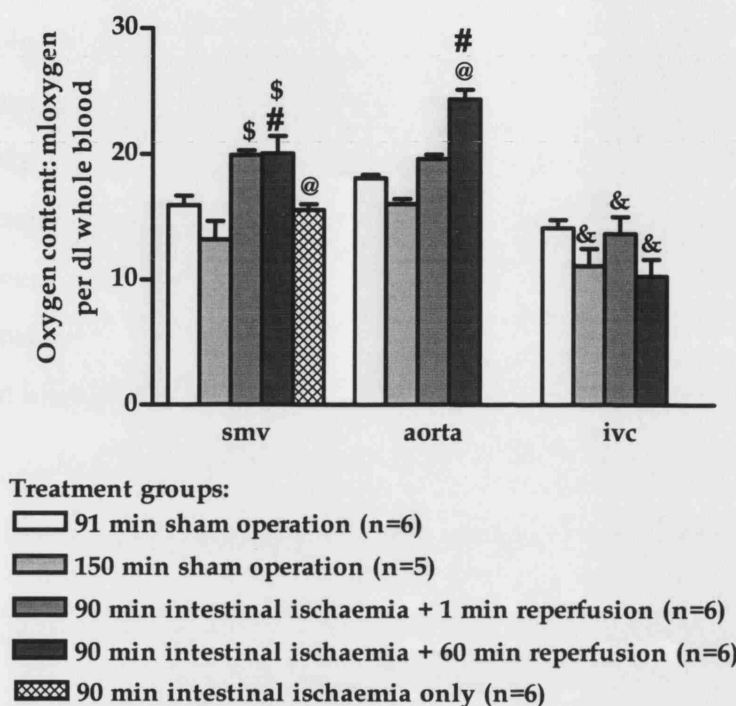


Figure 3.9: Whole blood oxygen content after IIR, IO or sham operation in the SMV, aorta and IVC.

\$ $P < 0.05$ versus IVC for the same treatment group

$P < 0.05$ versus 150 min sham operation for the same sample site

@ $P < 0.05$ versus 90 min intestinal ischaemia + 1 min reperfusion for the same sample site

& $P < 0.05$ versus aorta for the same treatment group

Interaction between treatment group and sample site: $P < 0.0001$

Figure 3.9 shows that whole blood oxygen content changed after different treatments depending on the sample site. In the SMV, oxygen content increased after 1 minute of reperfusion compared to 90 minutes of intestinal ischaemia only. Whole blood oxygen content in the SMV was not significantly different after 1 minute of reperfusion compared to 91 minutes of sham operation, or compared to aortic blood after 1 minute of reperfusion. After 60 minutes of reperfusion, blood

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in the SMV carried more oxygen than after 150 minutes of sham operation, and the same amount of oxygen as aortic blood after 60 minutes of reperfusion. In the aorta, whole blood oxygen content increased after 60 minutes of reperfusion only, to a level significantly higher than seen in the aorta after 1 minute of reperfusion or after 150 minutes of sham operation. In the IVC, whole blood oxygen content was significantly lower than in the aorta or in the SMV after 1 and 60 minutes of intestinal reperfusion. After 91 minutes of sham operation, whole blood oxygen contents were similar in the SMV, the aorta and the IVC. After 150 minutes of sham operation, IVC whole blood oxygen content was similar to SMV and lower than aortic levels.

A-V difference in whole blood oxygen content across the areas drained by the SMV and IVC after IIR or sham operation is shown in figure 3.10.

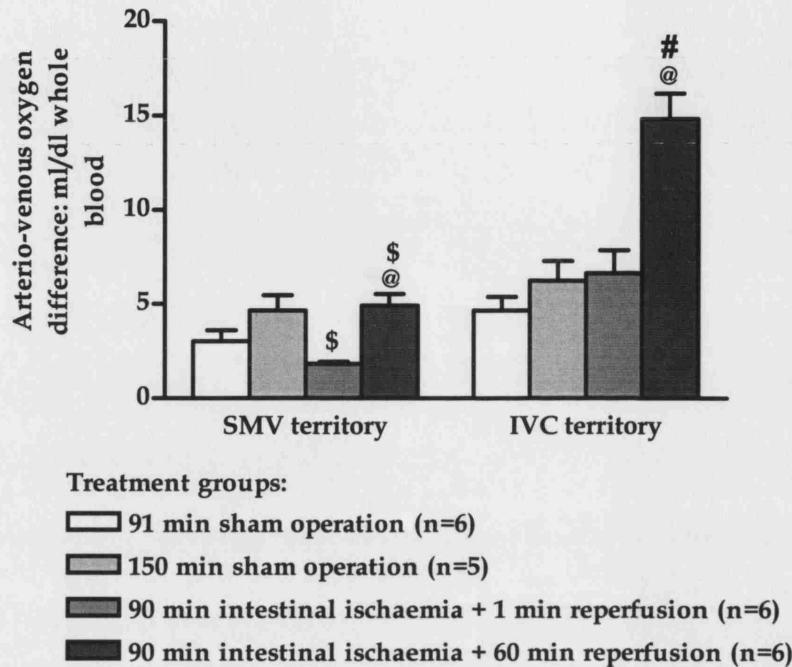


Figure 3.10: A-V difference in whole blood oxygen content after IIR or sham operation in the territories drained by the SMV or the IVC.

\$ $P < 0.05$ versus IVC for the same treatment group

@ $P < 0.05$ versus 90 min intestinal ischaemia + 1 min reperfusion for the same sample site

$P < 0.05$ versus 150 min sham operation for the same sample site

Interaction between treatment group and territory drained: $P < 0.0001$

Figure 3.10 demonstrates that the territories drained by the SMV and IVC exhibit dissimilar changes in A-V whole blood oxygen difference after IIR. The areas drained by the SMV and IVC have equivalent A-V whole blood oxygen differences after both 91 and 150 minutes of sham operation. After intestinal ischaemia followed by 1 minute of reperfusion, A-V whole blood oxygen difference is significantly lower than after 60 minutes of reperfusion in SMV-drained territory. After 60 minutes of reperfusion, A-V whole blood oxygen difference is significantly lower in territory drained by the SMV than in territory

drained by the IVC. In the territory drained by the IVC, A-V whole blood oxygen difference after 60 minutes of intestinal reperfusion rises to more than double that seen after reperfusion for 1 minute or after sham operation for 150 minutes.

Percentage change in whole blood oxygen content from aortic arterial supply to SMV and IVC drainage after IIR or sham operation is shown in figure 3.11.

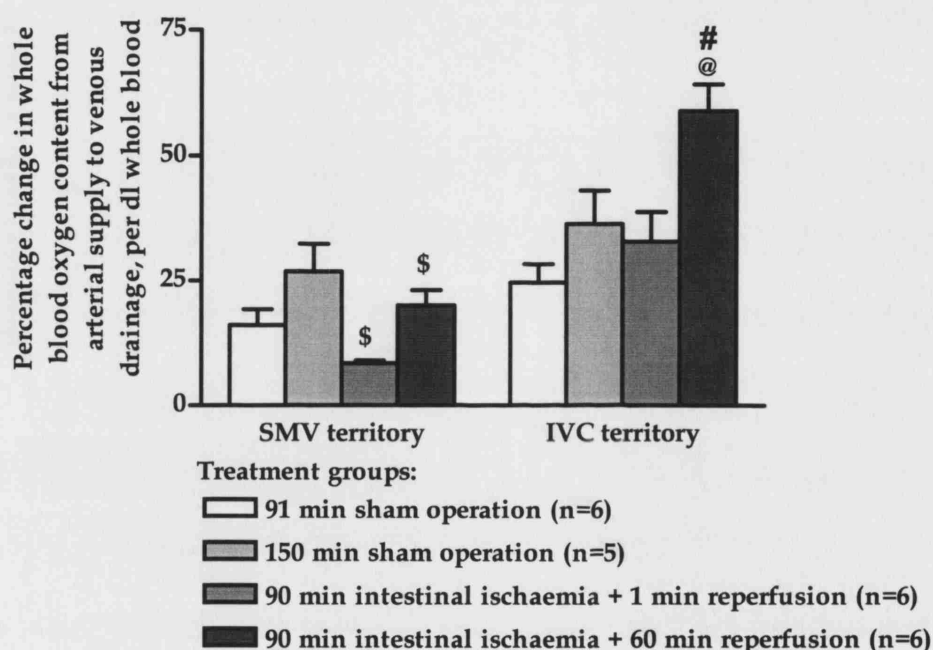


Figure 3.11: Percentage change in whole blood oxygen content from arterial supply to drainage via the SMV or the IVC after IIR or sham operation.

\$ $P < 0.05$ versus IVC for the same treatment group

@ $P < 0.05$ versus 90 min intestinal ischaemia + 1 min reperfusion for the same sample site

$P < 0.05$ versus 150 min sham operation for the same sample site

Interaction between treatment group and territory drained: $P < 0.0001$

Figure 3.11 shows that percentage change in whole blood oxygen content from arterial supply to venous drainage closely mirrors absolute A-V whole blood oxygen content difference (figure 3.10) in an unexpected manner. Identical oxygen whole blood content was delivered via the aorta to the territories drained by the

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SMV and the IVC in each treatment group. After sham operation, there is no difference in percentage change in whole blood oxygen content between the two territories. After IIR, however, the territory drained by the SMV extracts a very small percentage ($8.5 \pm 0.60\%$ [after 1 minute of reperfusion] and $20.1 \pm 3.03\%$ [after 60 minutes of reperfusion]) of delivered whole blood oxygen content – significantly less than that extracted in the territory drained by the IVC ($32.9 \pm 6.05\%$ [after 1 minute of reperfusion] and $59.1 \pm 5.30\%$ [after 60 minutes of reperfusion]). In the territory drained by the IVC, percentage change in whole blood oxygen content rose in response to prolonged intestinal reperfusion to significantly higher levels than that seen after reperfusion for 1 minute or sham operation for 150 minutes – this response was not seen in the territory drained by the SMV.

Discussion

Experiment 1: Plasma inorganic phosphate after intestinal ischaemia-reperfusion injury

Several investigators have reported elevated levels of Pi in systemic (Jamieson et al, 1979) and portal serum (Sodhi and Arora, 1993), and in peritoneal fluid (Jamieson et al, 1982), after IIR injury in both clinical (Delaney et al, 1999) and experimental studies. Other groups (Changani et al, 1998; Vejchapipat et al, 2001), have reported high levels of hepatic Pi after IIR – a possible indicator of increased Pi delivery to and accumulation within the liver after IIR injury. This study set out to test the hypothesis that excessive levels of Pi would be found in the SMV after IIR injury, and that this Pi would be removed by the liver.

This has been shown to be true in early IIR injury by the finding of higher than levels of Pi in the SMV but not in RA after 10 minutes of intestinal reperfusion compared to animals given sham operation only. At this time, presumably, the liver is successfully filtering out the increased Pi load from the intestines, preventing systemic Pi levels from rising. Later in reperfusion however the

situation changes markedly – SMV Pi levels return towards sham operation levels, and RA Pi levels rise to significantly greater than sham operation levels. The liver therefore not only fails to continue to filter out the Pi load arriving from the intestines, but appears to add to the systemic Pi load.

The origin of the raised Pi level in the SMV found in our plasma samples could be damaged intestinal cells as proposed by Taylor et al (Taylor et al, 1979), that gain access to the circulation by the increase in lumen-to-plasma permeability described after IIR by several groups (Langer et al, 1995; Sun et al, 2002; Yamagishi et al, 2002). Alternatively, leakage from red blood cells made more permeable by a toxic environment within reperfused bowel could contribute to the raised Pi levels seen in this experiment. This latter explanation is belied by the lack of correlation between free haemoglobin and Pi levels in the plasma samples demonstrated in this experiment, providing some evidence that red cell lysis has not contributed to plasma Pi load in either the SMV or RA.

The mechanism of the time-dependent failure of the liver to filter out SMV Pi is not clear. Liver metabolism is significantly deranged by IIR injury as demonstrated by numerous investigators (Fu et al, 1997; Turnage et al, 1991; Vejchapipat et al, 2001). Hepatic ATP falls, and there is evidence of peroxidation by reactive oxygen species (ROS) within the liver. Loss of ATP and cell damage by ROS may progressively compromise the liver's ability to assimilate Pi arriving from the liver thus preventing the liver from safely disposing of SMV Pi. The consumption of high energy phosphate metabolites generates Pi, and lipid peroxidation may release more Pi from phospholipid membranes. Cell death followed by lysis will also release Pi locally. These processes could increase the generation of Pi by the liver and contribute to the rise in systemic Pi seen late in intestinal reperfusion. High systemic levels of Pi would normally be excreted by the kidneys – urinary Pi levels were not checked in this experiment; therefore it is not possible to speculate further on the interaction between generation of excessive

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systemic Pi levels and possible failure to excrete Pi via the kidneys resulting in prolonged high systemic plasma Pi levels.

Experiment 2: Haematocrit, pH and oxygen carriage in the portal and systemic circulations after intestinal ischaemia-reperfusion injury

This experiment measured the effect of IIR on SMV, aortic and IVC haematocrit, pH and whole blood oxygen content in a rat model.

The overall pattern of effect on haematocrit and pH after IIR and sham operation was slightly surprising in that it had been expected that IIR would have a more profound effect on the composition of SMV blood, but the only difference in the pattern of effect found between the three sites sampled was an isolated rise in SMV haematocrit after one minute of intestinal reperfusion. By 60 minutes of intestinal reperfusion, all sites showed significant increases in haematocrit to levels that would be clinically important. The degree of haemoconcentration seen in this experiment would lead to increased blood viscosity and red cell sludging which might contribute to hepatic and other remote organ injuries and cause 'no re-flow' in reperfused intestine. The rapid transmission of haemoconcentration to aortic and IVC blood indicates the speed of the systemic response to IIR injury.

pH followed a similar pattern at all sites after both IIR injury and sham operation. pH fell after 60 minutes of intestinal reperfusion to levels well below the lower limit of normal, and this low pH would be expected to contribute to deranged local metabolism and to trigger other organs' (for example the lungs and kidneys) homeostatic mechanisms in an effort to correct this degree of acidosis. A pre-reperfusion sample was taken from the SMV only, and this showed a significant drop in pH with intestinal ischaemia only, which recovered to sham operation levels after only one minute of reperfusion. Again, the degree of change in pH is similar at all sites with no SMV bias seen, supporting the speed and strength of the systemic response to IIR injury indicated by the rise in haematocrit discussed above.

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Calculating whole blood oxygen content permitted an examination of oxygen content in the SMV, aorta and IVC, as well as a comparison of A-V difference in whole blood oxygen content between territories drained by the SMV and IVC after IIR and sham operation. Whole blood oxygen content was the same at all sites after sham operation for 91 minutes, and lower only in the IVC after 150 minutes. This was unexpected as it was assumed that oxygen content in the aorta would be higher than in the SMV and IVC at all times. In the SMV only, a pre-reperfusion sample was taken. Oxygen content in this sample (90 minutes of intestinal ischaemia) was equal to that after 91 minute sham operation – this was also unexpected. The maintenance of oxygen content after intestinal ischaemia for this length of time indicates very little oxygen extraction by ischaemic tissues and very little diffusion of oxygen out of stagnant blood into tissues. Collateral supply may have maintained oxygen content. After intestinal reperfusion, oxygen content in the SMV increased after one minute of reperfusion to become greater than IVC oxygen content. This level was maintained at 60 minutes of reperfusion, reaching a level significantly greater than after 150 minutes of sham operation in the SMV. The rise in SMV oxygen content compared to IVC levels after IIR injury indicates that the intestines are less able to remove oxygen from blood after IIR than the systemic periphery. Whole blood oxygen content in the SMV was equivalent to that in the aorta for all treatments given. This implies that the territory drained by the SMV extracts minimal oxygen under the conditions examined. This lack of oxygen extraction might be due to shunting of blood directly from arterioles to venules within the splanchnic circulation or to the 'short circuiting' of oxygen via the counter current mechanism in the intestinal villus (see Chapter 1, page 4). Aortic whole blood oxygen content exactly mirrors the changes in aortic haematocrit described above, indicating the expected pre-eminence of haemoglobin content in dictating whole blood oxygen content.

The A-V difference in whole blood oxygen content across the territories drained by the SMV and IVC was calculated both as absolute values and as

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percentage change in the whole blood oxygen content delivered to each area.

Absolute A-V difference in whole blood oxygen content demonstrates that after sham operation, A-V difference in oxygen content in both territories drained was equivalent. After IIR, the SMV territory extracted much less oxygen than the IVC territory. This may be due to shunting of blood or oxygen (via the counter-current mechanism in villi – see Chapter 1, page 4) in the intestines preventing extra extraction of oxygen. It is surprising that the intestines do not appear to extract any extra oxygen to compensate for recent hypoxia and build up ATP stores again despite the shunts described above. This may be due to a large volume of dead cells unable to use oxygen, or to local inhibition of oxygen extraction after IIR caused by local metabolic derangement that had not recovered at 1 minute of reperfusion. By 60 minutes of intestinal reperfusion, perfusion pressures are low, so oxygen should have sufficient time in a sluggish milieu to diffuse into tissues. A prolongation of this experiment to examine oxygen extraction with longer periods of intestinal reperfusion may differentiate between these possibilities. The marked increase in A-V difference in whole blood oxygen content indicating increased oxygen extraction in the systemic periphery drained by the IVC provides more evidence of the systemic response to IIR injury. The systemic periphery was not deprived of oxygen during this experiment, but did increase extraction of oxygen per decilitre of whole blood after 60 minutes of intestinal reperfusion. This is likely to be a reflection of relative stagnation of blood within the systemic circulation after 60 minutes of intestinal reperfusion. By this point, systemic blood pressure is low, heart rate is high and peripheral perfusion is sluggish. High extraction of oxygen under these circumstances is due to increased diffusion gradients into tissues and increased time within capillaries for gas exchange. An examination of oxygen extraction by peripheral tissue may differentiate between maintenance of normal levels of tissue oxygen extraction with sluggish oxygen supply and increased oxygen extraction after a major systemic insult.

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Percentage change in whole blood oxygen content mirrors absolute A-V difference in whole blood oxygen content. The areas drained by the SMV and IVC had similar percentage changes in whole blood oxygen content between arterial supply and venous drainage after sham operation for 91 and 150 minutes. These values agree with the published literature, being between 20 and 30%. After IIR injury, percentage change in whole blood oxygen content drops in the area drained by the SMV compared to that drained by the IVC, but does not change compared to percentage change after sham operation in the area drained by the SMV. This indicates a failure of the area drained by the SMV to increase percentage oxygen extraction to repay the oxygen debt incurred by 90 minutes of intestinal ischaemia. Again, this may be due to shunting of blood or oxygen preventing increased extraction of oxygen, or a local inability to increase oxygen release into tissues after IIR injury. After 60 minutes of intestinal reperfusion, percentage oxygen extraction in the area drained by the IVC increased to nearly 60% - this approaches the maximum oxygen extraction given in the literature (Samsel and Schumacker, 1994). This is likely to reflect the sluggish nature of the peripheral circulation after 60 minutes of intestinal reperfusion, and demonstrates that the peripheral tissues can access the oxygen delivered to maintain local oxygen tensions.

Summary and Conclusions

Intestinal ischaemia and reperfusion caused significant changes in the composition of the blood in both the portal and systemic circulations.

Plasma Pi levels were differentially altered in superior mesenteric and systemic venous blood after IIR. Levels were high early in reperfusion in the SMV and normalised by 60 minutes of intestinal reperfusion; Pi was initially normal in the RA but rose significantly with increasing intestinal reperfusion. This may be due to the induction of hepatic injury by IIR in this model causing a failure in the ability of the liver to remove an excessive Pi load delivered via the SMV.

Alternatively, the injured liver may begin to generate Pi and release it into the systemic circulation.

Haematocrit rose and pH fell in both SMV, aortic and IVC blood samples after IIR. This is likely to be due to the loss of plasma volume through altered endothelial and epithelial permeability after IIR causing haemoconcentration, and to reduced tissue perfusion causing a switch to anaerobic metabolism and the generation of lactic acid respectively. Oxygen extraction in the territories drained by the SMV and IVC behave differently after intestinal reperfusion. Territory drained by the SMV maintained an A-V difference in whole blood oxygen content similar to that after sham operation, therefore failing to extract more oxygen from arterial blood than after sham operation. This may be due to a combination of gross shunting of blood from arterioles to venules, and specific shunting of oxygen via the villus counter-current mechanism in perfused villi, or to the presence of many dead or IIR-injured metabolically inactive cells unable to extract oxygen. Territory drained by the inferior vena cava did not increase the A-V difference in whole blood oxygen content after one minute of intestinal reperfusion over a level equivalent to sham operation, but by sixty minutes of intestinal reperfusion A-V difference in whole blood oxygen content and therefore oxygen extraction had risen markedly and was much higher than after sham operation for 150 minutes, and in the territory drained by the SMV after 60 minutes of reperfusion. This may be explained by sluggish flow of viscid blood allowing maximum oxygen extraction by the (functional) systemic periphery.

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Chapter 4: The effects of whole-body hypothermia on liver inflow, blood composition and hepatic metabolism after intestinal ischaemia-reperfusion

Introduction

Intestinal ischaemia restricts liver inflow by reducing portal venous flow. During intestinal ischaemia, the proportion of liver inflow arriving via the portal vein decreases from the normal resting level of 70 – 80% (Clemmesen et al, 1999; Jiao et al, 2000). This effect is exaggerated by a reflex increase in hepatic arterial flow – the ‘hepatic arterial buffer response’ – thought to be due to decreased washout of local adenosine resulting in local hepatic arterial vasodilatation (Lautt et al, 1985). The liver is relatively hypoperfused during intestinal ischaemia [i.e. total hepatic inflow rate falls] (Turnage et al, 1996), but if hepatic arterial inflow is sustained or increased, the liver is unlikely to become hypoxic as absolute oxygen delivery is maintained. Intestinal reperfusion is followed initially by restoration of intestinal outflow (Myers and Hernandez, 1992) and occasionally local hyperaemia (Savas et al, 1997), restoring portal venous inflow into the liver. Several studies have shown that after intestinal reperfusion intestinal blood flow is not sustained and a secondary fall in portal venous outflow is seen, resulting in late hepatic hypoperfusion (Myers and Hernandez, 1992; Turnage et al, 1996). The cause of the late failure of intestinal reperfusion is not clear, and may be related to post-reperfusion increases in mesenteric resistance (Oktar et al, 2002), hyperviscosity of blood (Yamamoto et al, 2001), increased back-pressure from non-perfused sinusoids in the liver (Horie et al, 1996), or global low-flow due to systemic arterial hypotension (Aneman et al, 1996).

Studies on the effect of intestinal ischaemia-reperfusion (IIR) on hepatic inflow also demonstrate a large sham operation effect (Nakamura et al, 2001; Turnage et al, 1996) with decreases in both portal venous and hepatic arterial blood flow with prolonged general anaesthetic. The reasons for this are not clear but may be related to a decrease in splanchnic perfusion during the mild stress of general anaesthesia or a direct effect of general anaesthetic on mesenteric vascular tone. In un-resuscitated animal models, relative hypovolaemia over a prolonged experiment may be important and could also exaggerate the effect of IIR on hepatic inflow, as well as contribute to the effects observed in sham operated animals.

The majority of studies examining hepatic injury after IIR demonstrate that hepatic injury is not apparent until after intestinal reperfusion (Changani et al, 1998; Savas et al, 2003; Turnage et al, 1996; Vejchapipat et al, 2001). Therefore it is not likely to be hypoperfusion of the liver during intestinal ischaemia that results in hepatic injury after IIR, but possibly factors released by the reperfused intestines (acting either locally or globally) or the late hepatic hypoperfusion seen after prolonged intestinal reperfusion, in which the 'hepatic arterial buffer response' is less likely to be seen [possibly due to the new, toxic milieu in the liver] (Turnage et al, 1996). Bioactive factors released by the intestines after IIR include chemokines (Yagihashi et al, 1998), cytokines (Squadrito et al, 1997), vasoactive peptides (Oktar et al, 2002) and endotoxin (Yamamoto et al, 2001). Activated leukocytes from the mesenteric vascular bed are carried to the liver in portal venous blood (Horie et al, 1996) and can release further bioactive molecules within hepatic tissue if trapped in hepatic sinusoids. Fixed tissue macrophages in the liver (Kupffer cells) are rapidly activated after IIR and propagate the inflammatory reaction to IIR (Towfigh et al, 2000). Thus the inflammatory response to IIR rapidly becomes systemic, and splanchnic organs are exposed to inflammatory molecules released from other organs, resulting in a positive feedback loop encouraging further release of inflammatory factors from the intestines.

IIR has been shown to have a significant impact on blood composition: haematocrit increases (Yamamoto et al, 2001) and pH falls (Takeyoshi et al, 1999) [see also Chapter 3 of this thesis: pages 145 - 177]. Despite a rise in whole blood oxygen content in portal and systemic venous blood, oxygen consumption by the intestines falls (Nankervis et al, 2000), largely due to failure of mesenteric inflow. The effects of these changes on hepatic inflow and metabolism after IIR have not been examined in isolation, but several mechanisms of action may be hypothesized. Increased haematocrit could encourage red cell sludging in hepatic sinusoids reducing the efficiency of trans-hepatic blood flow (Horie et al, 1996; Ishimura et al, 2002). Lowered pH may affect the enzymatic milieu within the liver, altering the hepatic metabolic response to IIR (Holecek et al, 2003). Changes in hepatic oxygen delivery could result in maintenance of hepatic function after IIR or damage the liver by the production of reactive oxygen species (Hoek and Pastorino, 2002; Mitsuyoshi et al, 1999; Younes et al, 1989). Any intervention beneficial to the maintenance of normal blood composition could also reduce the impact of IIR on hepatic inflow and metabolic derangement after IIR.

IIR adversely affects hepatic metabolism resulting in hepatic energy failure (Vejchapipat et al, 2001), release of hepatic transaminases (Fu et al, 1997), onset of insulin resistance (Ma et al, 2003) and reduction in bile production (Nakamura et al, 2001). The effect of compromised hepatic inflow on these observations has been examined in isolated dual organ preparations allowing the control of hepatic inflow and oxygen supply (Poggetti et al, 1992). It was found that bile flow decreased after IIR even in the absence of increased haematocrit, compromised hepatic inflow, leukocytes and abnormal oxygen delivery, suggesting a direct effect of bioactive factors in intestinal outflow on the liver. It is hypothesized that interventions beneficial to hepatic inflow and normalisation of blood composition after IIR could also normalise hepatic metabolism but this may not be observed if bioactive factors released by the intestines are not also reduced.

Hypothermia has been used to reduce the impact of circulatory arrest (Cooper et al, 2000), head injury (Dietrich et al, 1994) and IIR-induced local (Kalia et al, 2002) and remote injury (Gundersen et al, 2001). The mechanisms behind the beneficial effects of hypothermia have not been clearly defined and may include leukocyte stabilisation, inhibition of selected enzymes altering intra-cellular homeostasis (Wang et al, 2000), metabolic rate (Schumacker et al, 1987) and gene transcription (Kato et al, 2002; Ning et al, 2002), induction of the heat shock response (Cullen and Sarge, 1997) and/or reduction in vascular permeability (Barone et al, 1997). It has been shown that whole-body hypothermia delays the onset of hepatic energy failure after IIR in a rat model (Vejchapipat et al, 2001), and attenuates the effect of hepatic ischaemia in a mouse model (Kato et al, 2002).

It is hypothesized that whole body hypothermia will be beneficial to hepatic inflow and/or blood composition and hepatic metabolism after IIR. This is based on interpretation of the benefits of hypothermia described above, and the known effects of IIR on these parameters, as described previously in this introduction (impaired hepatic perfusion, raised haematocrit, lowered pH, hepatic energy failure). It is hypothesized that whole body hypothermia could reduce the impact of IIR on total hepatic inflow (THI) and the cardiovascular parameters measured. Changes in haematocrit, pH, whole blood oxygen content, and arterio-venous (A-V) difference in whole blood oxygen content in both portal and systemic circulations may be abrogated. Hypothermia could also reduce hepatic metabolite derangement after IIR, and change the relationship of hepatic tissue metabolite levels to THI at the end of the experiment (i.e. immediately prior to liver tissue sampling). A detailed analysis of the effects of whole body hypothermia during IIR on these parameters may allow some insight into the gross mechanism of any beneficial action of hypothermia demonstrated.

The primary aim of this experiment was to document the changes in heart rate, arterial blood pressure and liver inflow in real time during IIR and sham

operation at normothermia, and contrast these with changes recorded during IIR and sham operation with whole body hypothermia in a rat model.

Secondary aims were: 1) to measure haematocrit, pH, whole blood oxygen content, and A-V difference in whole blood oxygen content in the portal and systemic circulations after IIR, and to compare the impact of IIR at normothermia and hypothermia relative to sham operated controls at each temperature; and 2) to examine the effect of IIR at normothermia and hypothermia on selected hepatic tissue metabolites, correlating the concentration of these metabolites with THI at the end of intestinal reperfusion (i.e. immediately prior to tissue sampling) to explore the effect of whole-body hypothermia on these parameters and their relationship to each other.

Materials and Methods

Animal preparation

Age-matched adult male Sprague-Dawley rats (8 – 12 weeks old) weighing between 242g and 340g (median weight was 292g) were studied. Rats were kept under standardised conditions for food, water, light and temperature. All animals had access to standard rat chow and water *ad libitum*. Animal numbers reported below refer to animals completing the flow part of the experimental protocol.

There were four experimental groups:

- A) Normothermic sham operation for 180 minutes (n=8)
- B) Normothermic intestinal ischaemia-reperfusion (30 minutes run-in + 90 minutes ischaemia + 60 minutes reperfusion) (n=12)
- C) Hypothermic sham operation for 180 minutes (n=10)
- D) Hypothermic intestinal ischaemia-reperfusion (30 minutes run-in + 90 minutes ischaemia + 60 minutes reperfusion) (n=11)

Rectal temperature was monitored continuously immediately after induction of deep surgical anaesthesia (absence of response to firm web-space

pinch) and hypothermic groups were allowed to cool passively under general anaesthetic until the target temperature ($32 \pm 0.5^{\circ}\text{C}$) was achieved prior to the start of the experiment. Normothermic rats were maintained at $37 \pm 0.5^{\circ}\text{C}$ and hypothermic rats at $32 \pm 0.5^{\circ}\text{C}$ using a heating pad and radiant lamp for the duration of the experiment.

Surgical procedure

Animals were anaesthetised with 3% halothane by inhalation in a 50:50 mixture of oxygen and nitrous oxide at a flow rate of 200ml/min per gas until deep surgical anaesthesia had been induced. Anaesthesia was maintained using 0.8-1.5% halothane in a 50:50 mixture of oxygen and nitrous oxide at the same flow rates for the remainder of the experiment. An arterial line was positioned in the right common femoral artery and flushed with 0.5ml of heparinised saline (25 units lithium heparin salt (Sigma Chemical Co, St Louis, MO, USA) per ml). After calibration with a sphygmomanometer, the arterial line was used to measure arterial blood pressure via a transducer (TranspakIV Abbott Pressure Transducer, Abbott Laboratories, Chicago, IL, USA) connected to the same Transonic® flow-meter (T206 Transonic® Animal Research Flowmeter, Transonic Systems Inc., Ithaca, NY, USA) used to record flow in the portal vein and hepatic artery. All animals had midline laparotomy and exposure of the superior mesenteric artery (SMA). A sling of 3/0 nylon was placed around the SMA in Group B and D animals, taking care not to alter flow in the SMA when the abdominal contents were returned to the abdominal cavity. A custom-made forked semi-rigid plastic plate was sited between the diaphragm and liver in all animals. This was held in place by clamping it to the xiphoid process of the sternum with a small haemostat. It served to reduce the movement of the porta hepatis due to respiratory excursions, without restricting the flow in the hepatic veins, inferior vena cava or aorta due to the forked design, which fitted around these blood vessels. The porta hepatis was exposed and the portal vein cleared of fat. A 1.5mm R Transonic®

transit-time ultrasound (TTUS) flow probe (Transonic Systems Inc., Ithaca, NY, USA) was sited around the portal vein such that flow was not restricted by the probe. The common biliary duct was divided to improve access to the hepatic artery. The hepatic artery was dissected free and cleaned of fat. A 0.7mm V Transonic® TTUS flow probe (Transonic Systems Inc., Ithaca, NY, USA) was sited around the hepatic artery and adjusted to give an acceptable trace without restricting flow in the artery. Acoustic coupling within the flow probes was facilitated by application of water-based sterile jelly (H.R. Lubricating jelly, Carter Products, Division of Carter-Wallace, Inc., New York, NY, USA) around the blood vessels. Flow and blood pressure were recorded using a T206 Transonic® Animal Research Flowmeter (Transonic Systems Inc., Ithaca, NY, USA).

Recording was started when satisfactory traces had been established from both flow probes and the blood pressure transducer. Set-up time was between 25 and 75 minutes (median time was 35 minutes). In Groups A and C (sham operation), portal venous flow (PVF), hepatic arterial flow (HAF) and blood pressure were recorded for 180 minutes with no further surgical intervention. In Groups B and D (IIR) PVF, HAF and blood pressure were recorded continuously during 30 minutes of 'run-in' (no surgical intervention) then 90 minutes of intestinal ischaemia effected by tightening the nylon sling around the SMA, and then 60 minutes of intestinal reperfusion effected by removing the nylon sling from the SMA. Efficacy of occlusion and de-occlusion of the SMA was confirmed by direct inspection of the arteries of the small bowel mesentery. Flow probe position was not affected by tightening or loosening the SMA sling.

At the end of the experiment a segment of the right lobe of the liver was rapidly resected and plunged into liquid nitrogen. The time from the beginning of the resection to immersion in liquid nitrogen was less than 5 seconds in all cases. The bleeding at the liver edge was controlled with a vascular clamp, and 0.5ml blood samples were taken from the superior mesenteric vein (SMV), inferior vena cava (IVC) and aorta as rapidly as possible. All blood samples were taken within 2

minutes of the liver resection. A 21G Microlance®3 needle and 1ml Plastipak® syringe, both from Becton, Dickinson UK Ltd, Cowley, Oxford, UK) were used to take each blood sample. Needle and syringe were flushed with heparinised normal saline (25 units heparin lithium salt (Sigma Chemical Co, St Louis, MO, USA) per ml) immediately prior to use.

Flow and blood pressure monitoring

Flow and blood pressure were recorded using WinDaq software (Dataq Instruments, Akron, Ohio, USA) at a sample rate of 100 data points per second. This system yielded excellent waveforms in all traces (see figure 4.1).

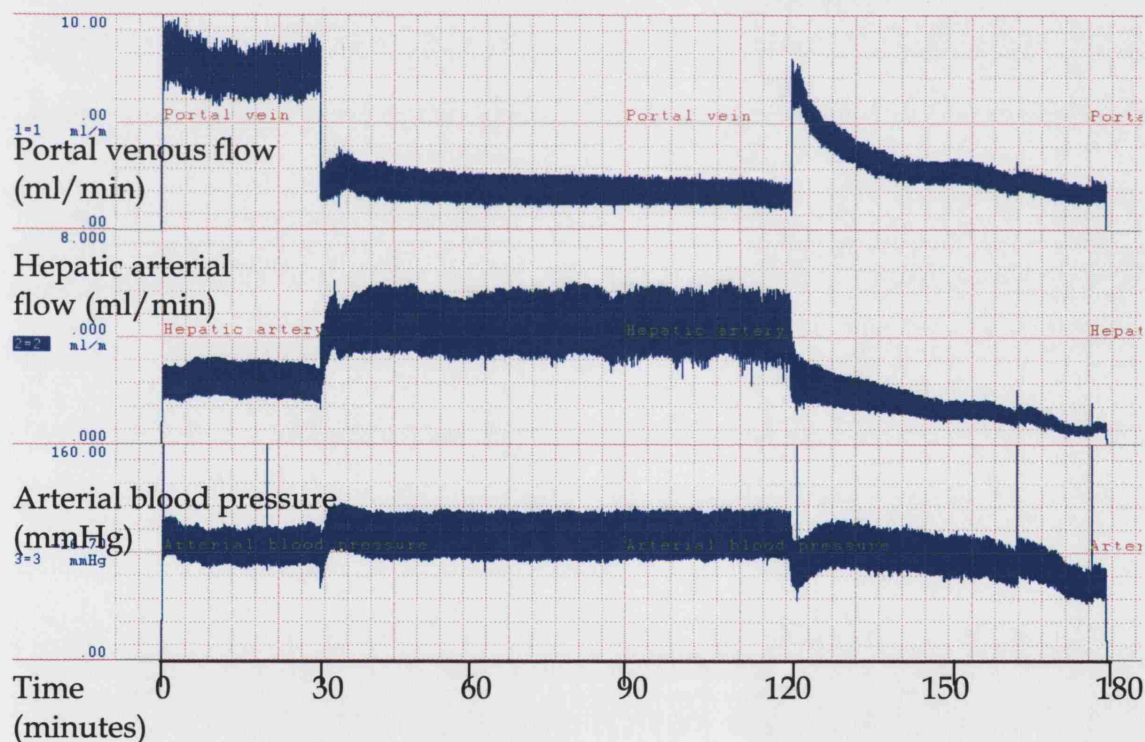


Figure 4.1: Example of entire experimental recording from an animal in group B (normothermic IIR).

Advanced Cudas software (Dataq Instruments, Akron, Ohio, USA) was used to select peaks in the blood pressure waveform and to calculate heart rate. Data was exported into Microsoft Excel 2002 (Microsoft Office XP Professional

with Publisher version 2002, Microsoft Corporation, Redmond, Washington, USA) for display and analysis. Mean arterial blood pressure (MAP) was calculated as the numerical mean of arterial blood pressure recorded. Arterial blood pressure was recorded 100 times per second, thus accurately reflecting the changing pattern of blood pressure with the cardiac cycle despite the rapid heart rate of the rat (up to 400 beats per minute in this experiment) according to the Nyquist criterion. The mean of each 100-second sample package was calculated by the WinDaq software and exported to Windows Excel for further summary into a one-minute mean for each animal for each experimental minute. Because this method of calculating mean arterial blood pressure used 100 data points per second covering the whole of the cardiac cycle, it is superior to the clinical method of approximating MAP by using diastolic plus one-third of systolic pressure readings. THI was calculated as the sum of portal venous and hepatic arterial flow. Individual animals' data were summarized to successive 60-second mean values over the whole experiment. Group mean values for each experimental minute were achieved by calculating the mean of each animal's 60-second mean value for that minute, which also yielded a standard error of the mean for the group for that minute.

Liver metabolite measurement

Liver samples were stored in aluminium foil at -80°C for 1 – 28 days (median storage time was 5 days) until metabolites were extracted using methanol/chloroform/water. Measurements were performed as described in Chapter 2 (pages 116 – 118).

Blood gas measurement

All blood samples were used immediately to fill the sample chamber of an EG7+ iSTAT® portable clinical analyser cartridge (iSTAT® Corporation, Princeton, NJ, USA), and analysed straight away using an iSTAT® portable clinical analyser (iSTAT® Corporation, Princeton, NJ, USA). The delay between initiation of sample

collection and analysis was less than 2 minutes in all cases. Measurements were performed as described in Chapter 3 (pages 154 – 155).

Statistical analysis

Data collected over the whole time-course of the experiment are presented as the group mean of successive 60 second periods \pm standard error of the mean (SEM) against experimental time [heart rate, MAP and blood flow (portal venous, hepatic arterial and total hepatic inflow)]. Summary variables were chosen to represent this data at critical time-points, and were the group mean value at the following time-points: 30th minute - last full minute of run-in; 32nd minute - first full minute of intestinal ischaemia; 120th minute - last full minute of intestinal ischaemia; 122nd minute - first full minute of intestinal reperfusion; 180th minute - last full minute of intestinal reperfusion – see figure 4.2.

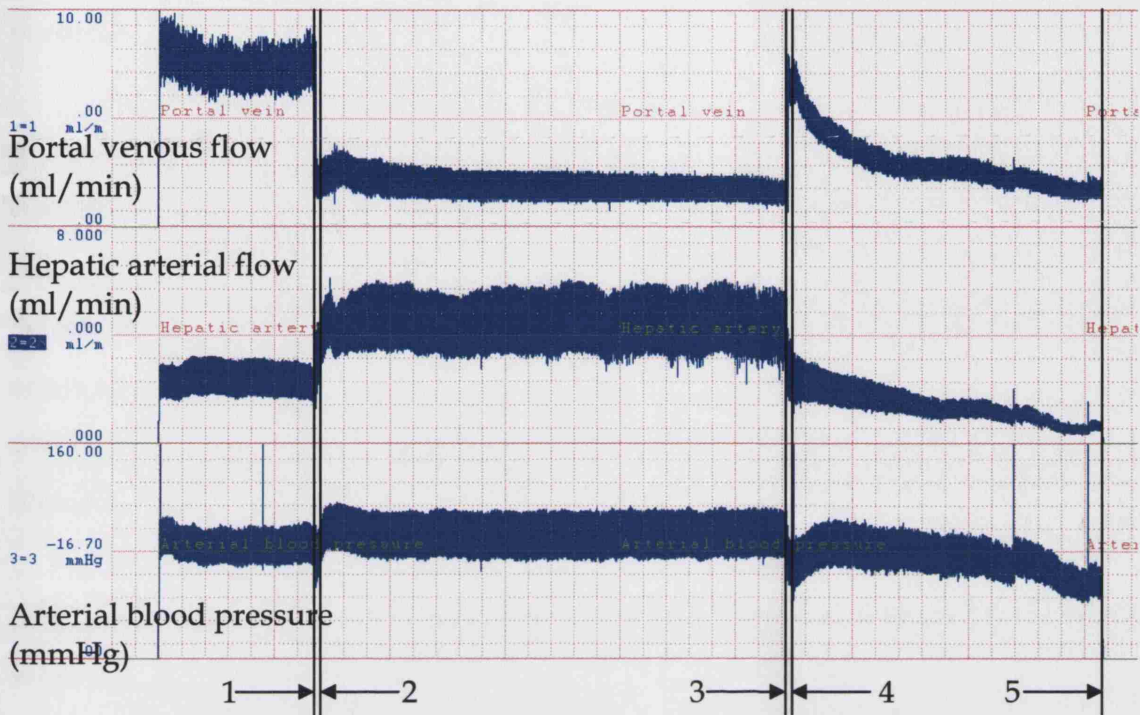


Figure 4.2: Summary parameter time-points identified on a whole experimental recording from an animal in group B (normothermic IIR).

- 1 = 30th minute (last full minute of run-in)
- 2 = 32nd minute (first full minute of intestinal ischaemia)
- 3 = 120th minute (last full minute of intestinal ischaemia)
- 4 = 122nd minute (first full minute of intestinal reperfusion)
- 5 = 180th minute (last full minute of intestinal reperfusion)

Summary parameter data are presented as group mean \pm SEM. After testing for normality of data, within treatment group statistical analysis was performed by comparison of summary variables using repeated measures ANOVA with Tukey's multiple post hoc comparisons, and examination of linear trend to look for change in summary variables over experimental time. Between treatment groups statistical analysis was performed by ANOVA with Tukey's multiple post-hoc comparison unless variance was not equivalent, when multiple unpaired t-

tests with Welch's correction for unequal variance were used with Bonferroni correction for repeated measurements.

Data collected at the end of the experiment [(arterial blood gas measurements – haematocrit, pH, blood oxygen content and drop in blood oxygen content)] are presented as group mean \pm SEM. After testing for normality of data, between and within experimental groups statistical analysis was performed by ANOVA with Tukey's multiple post hoc comparisons unless variance was not equivalent, when multiple paired and unpaired t-tests were used with Welch's correction for unequal variance and Bonferroni correction for repeated measurements.

Liver metabolite data are shown as the group mean \pm SEM for each treatment group. After testing for normality of data, between group comparisons were made using ANOVA with Tukey's multiple post-hoc tests unless variance was not equivalent, when multiple unpaired t-test's with Welch's correction for unequal variance was used with Bonferroni correction for multiple measurements. Significant correlation between concentration of liver tissue metabolites and THI in the final minute of the experiment for each treatment group were examined using linear regression.

Statistical significance was set at $P < 0.05$ after multiple measurements had been taken into account. P values are quoted after correction for multiple comparisons, unless stated otherwise. All statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, CA, USA).

Results

Data recording using WinDaq acquisition software was of consistently high quality, and demonstrated the rapidity of effect of SMA occlusion (figure 4.3)

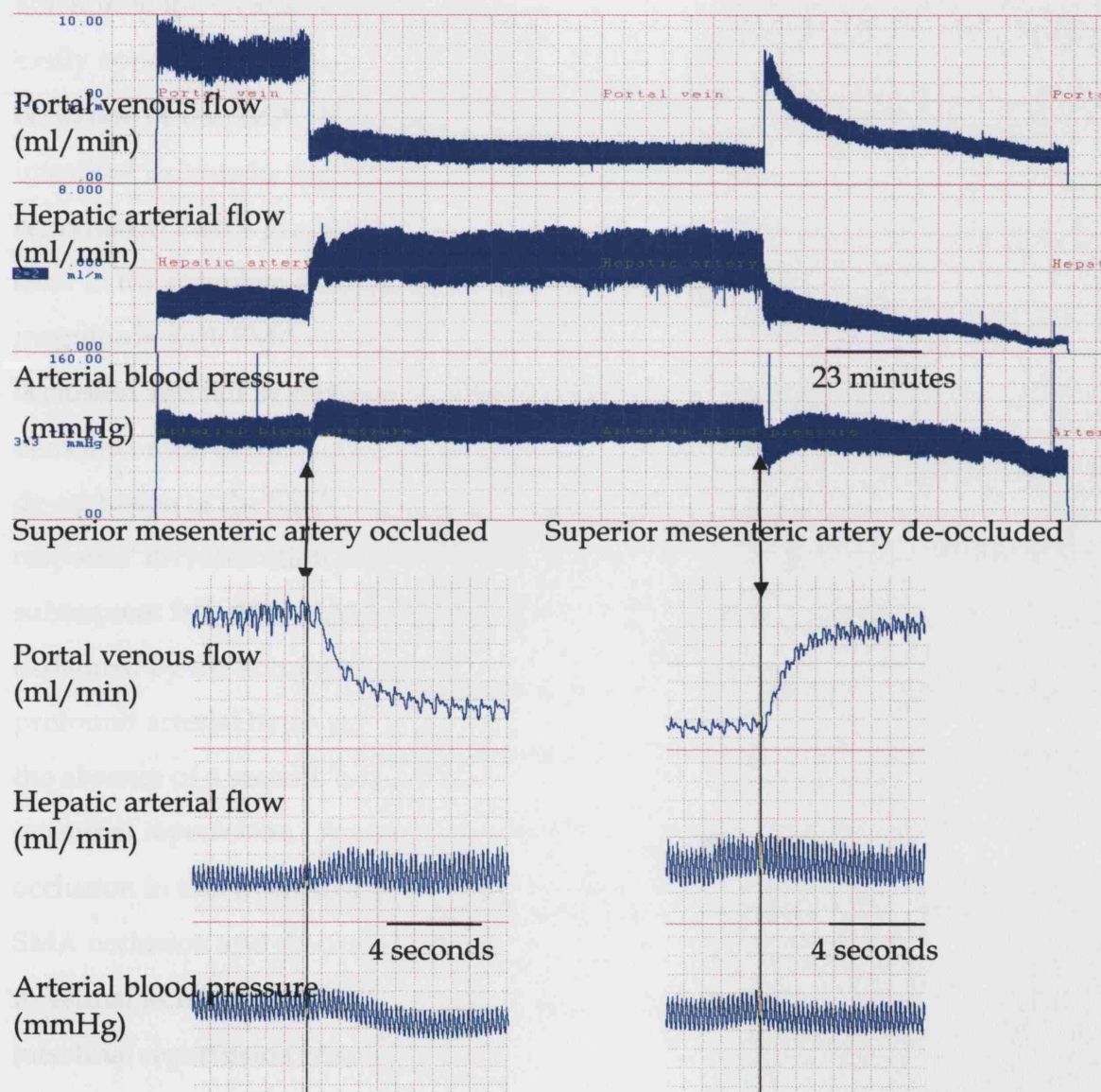


Figure 4.3: Sample of data recorded using WinDaq acquisition software
 Top: whole experimental recording of rat in group B (normothermic IIR)
 Bottom: enlarged sections showing rapidity of effect of superior mesenteric artery occlusion and de-occlusion on portal venous flow, hepatic arterial flow and arterial blood pressure.

Figure 4.1 shows a complete experimental trace (PVF, HAF, arterial blood pressure) from a single animal undergoing normothermic IIR. This treatment group was chosen for detailed discussion as it represents the injury group (IIR) without therapeutic intervention (i.e. without whole body hypothermia to ameliorate the effects of IIR). The rapid fall-off in PVF after SMA occlusion is easily appreciated in the enlarged section, as is the rapid restoration of PVF on de-occlusion of the SMA. The larger picture of a sustained suppression of PVF during intestinal ischaemia, followed by full restoration of flow immediately on intestinal reperfusion then a gradual fall-off in PVF with increasing experimental time is clear in the complete experimental trace. HAF undergoes less dramatic changes in magnitude with SMA occlusion and de-occlusion. The increase in HAF after SMA occlusion and fall in PVF is clear in the complete experimental trace. This is a demonstration of the 'hepatic arterial buffer response'. The rapid drop in HAF on de-occlusion of the SMA represents the reverse of the 'hepatic arterial buffer response' on restoration of normal PVF in early intestinal reperfusion. The subsequent fall-off in HAF with prolonged intestinal reperfusion is not entirely explained by the accompanying drop in arterial blood pressure, and results in profound arterial hypo-perfusion of the liver by the end of the experiment. Note the absence of a second 'hepatic arterial buffer response' with declining PVF late in intestinal reperfusion. Arterial blood pressure reacted to SMA occlusion and de-occlusion in this treatment group with transient decreases in mean pressure after SMA occlusion and de-occlusion (more prolonged on de-occlusion). Prolonged intestinal ischaemia resulted in a widening of pulse pressure and prolonged intestinal reperfusion resulted in a gradual decline in MAP and a restoration of initial pulse pressure.

Heart rate, blood pressure and flow measurements

Heart rate (group data: 60 second group mean \pm SEM over experimental time) during IIR or sham operation at normothermia and hypothermia is shown in figure 4.4.

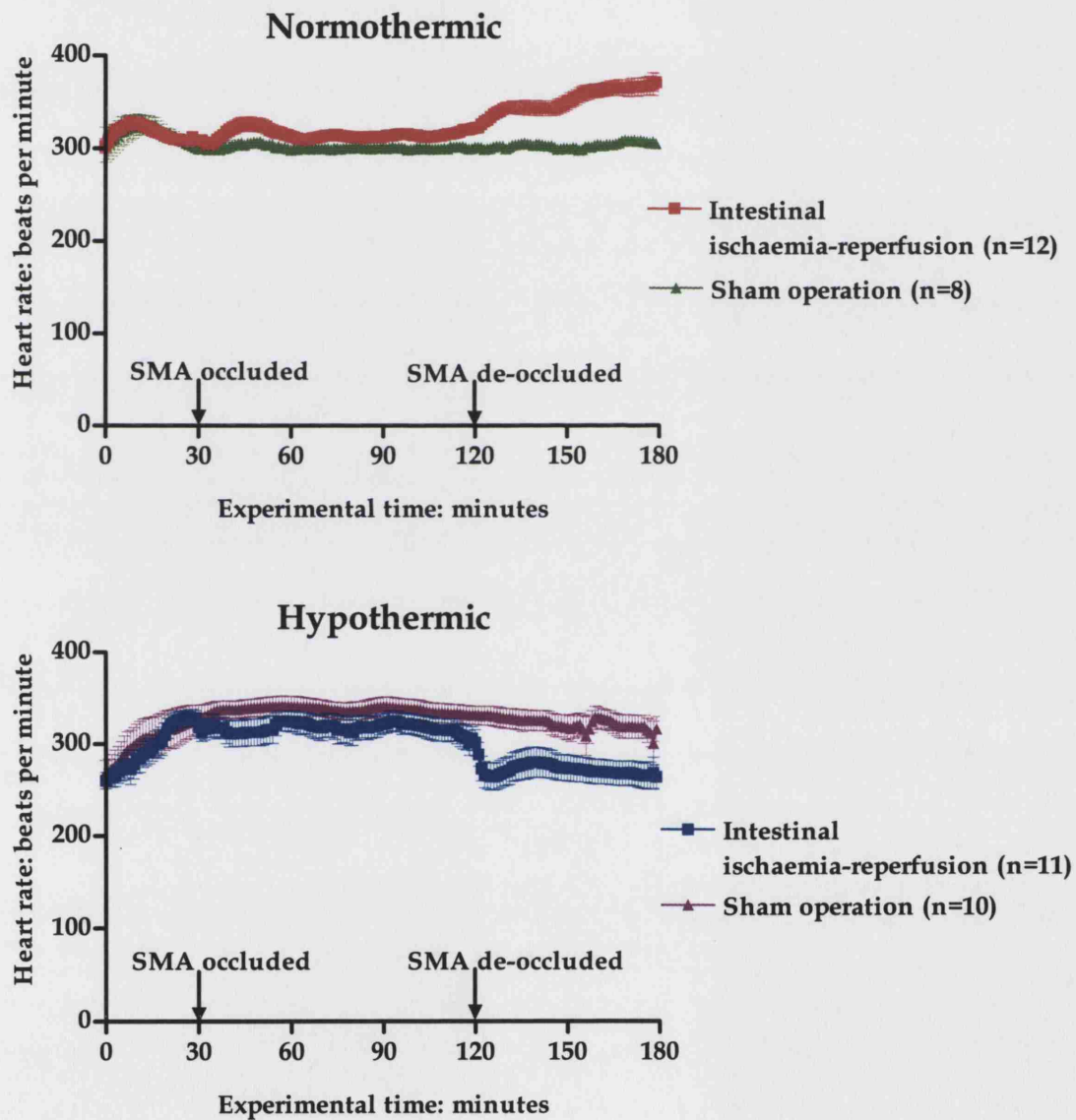


Figure 4.4: Heart rate during IIR and sham operation in normothermic and hypothermic rats: group data, 60-second group mean \pm SEM

Overall, normothermic IIR resulted in a gradual increase in heart rate over experimental time, most of which occurred during intestinal reperfusion. Normothermic and hypothermic sham operation showed no significant change in heart rate with experimental time. Hypothermic IIR-treated rats showed a sustained drop in heart rate, which is most marked on intestinal reperfusion.

Comparison of summary measurement points for heart rate within and between treatment groups is shown in figure 4.5.

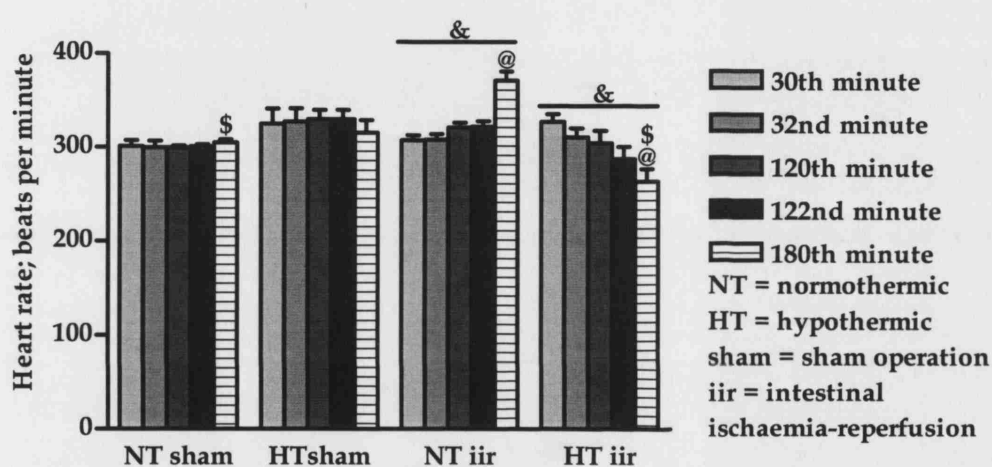


Figure 4.5: Summary measurements of heart rate during IIR and sham operation in normothermic and hypothermic rats.

@ $P < 0.05$ versus 30th minute, same treatment

\$ $P < 0.05$ versus normothermic IIR, same time-point

& $P < 0.05$ (change over experimental time)

No significant change in heart rate was seen in either sham operation group. Normothermic IIR rats showed a large increase in heart rate measured at the 180th minute. This is significantly greater than the heart rate at the 30th minute in normothermic IIR rats and significantly greater than the heart rate of hypothermic IIR and normothermic sham operation treated rats in the 180th minute. Hypothermic IIR rats showed a gradually decreasing heart rate over the whole

Chapter 4: The effects of whole-body hypothermia on liver inflow, blood composition and hepatic metabolism after intestinal ischaemia-reperfusion

experiment, finishing with a rate in the 180th minute significantly lower than that in the 30th minute.

MAP group data (60 second group mean \pm SEM over experimental time) during IIR or sham operation at normothermia and hypothermia is shown in figure 4.6.

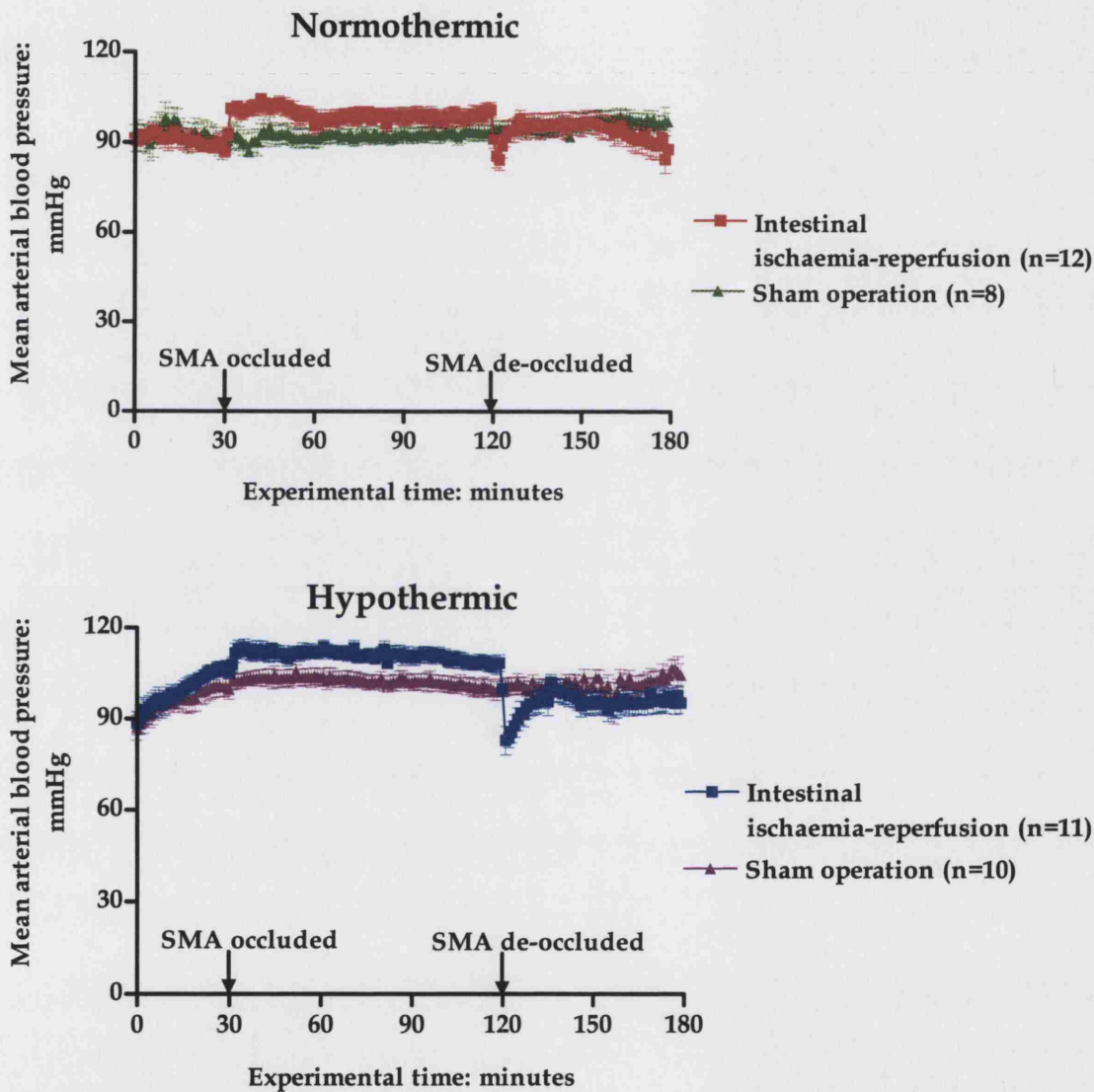


Figure 4.6: MAP during IIR and sham operation in normothermic and hypothermic rats: group data, 60-second group mean \pm SEM

Overall, MAP responded to sham operation at both normothermia and hypothermia with a small increase over experimental time. IIR resulted in a small rise on SMA occlusion and a small drop on SMA de-occlusion that is not sustained

in either the normothermic or the hypothermic groups. Normothermic IIR rats showed a late decrease in MAP towards the end of intestinal reperfusion – this was not seen in hypothermic IIR rats.

Comparison of summary measurement points for MAP within and between treatment groups is shown in figure 4.7.

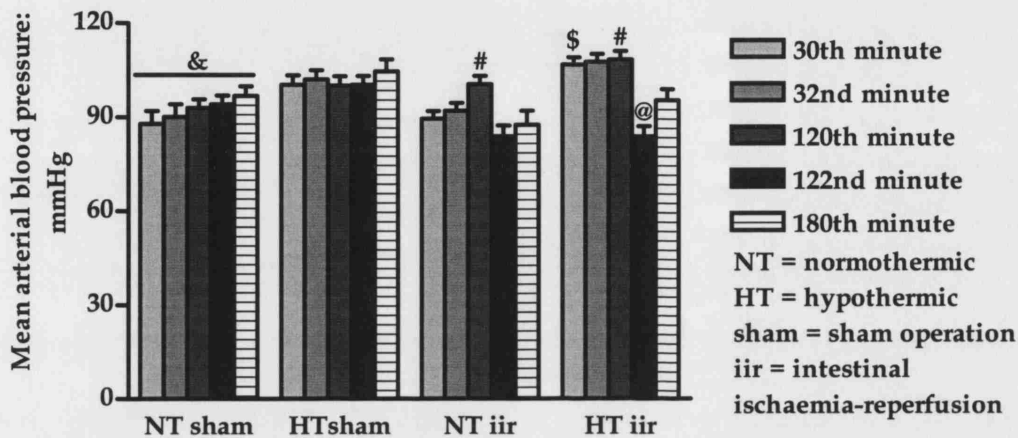


Figure 4.7: Summary measurements of MAP during IIR and sham operation in normothermic and hypothermic rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus NT iir, same time point

& $P < 0.05$ (change over experimental time)

A small but significant rise in MAP over the whole experiment was shown during normothermic sham operation, but not during hypothermic sham operation. A significant fall in MAP was noted in the normothermic IIR group between the 120th and 122nd minute – i.e. immediately after intestinal reperfusion. Hypothermic IIR rats had a higher MAP at the 30th minute than normothermic IIR rats and also showed a significant drop in MAP between the 120th and 122nd minute. MAP in this group was lower than in the 30th minute at the 122nd minute, but had recovered by the 180th minute.

PVF group data (60 second group mean \pm SEM over experimental time) during IIR or sham operation at normothermia and hypothermia is shown in figure 4.8.

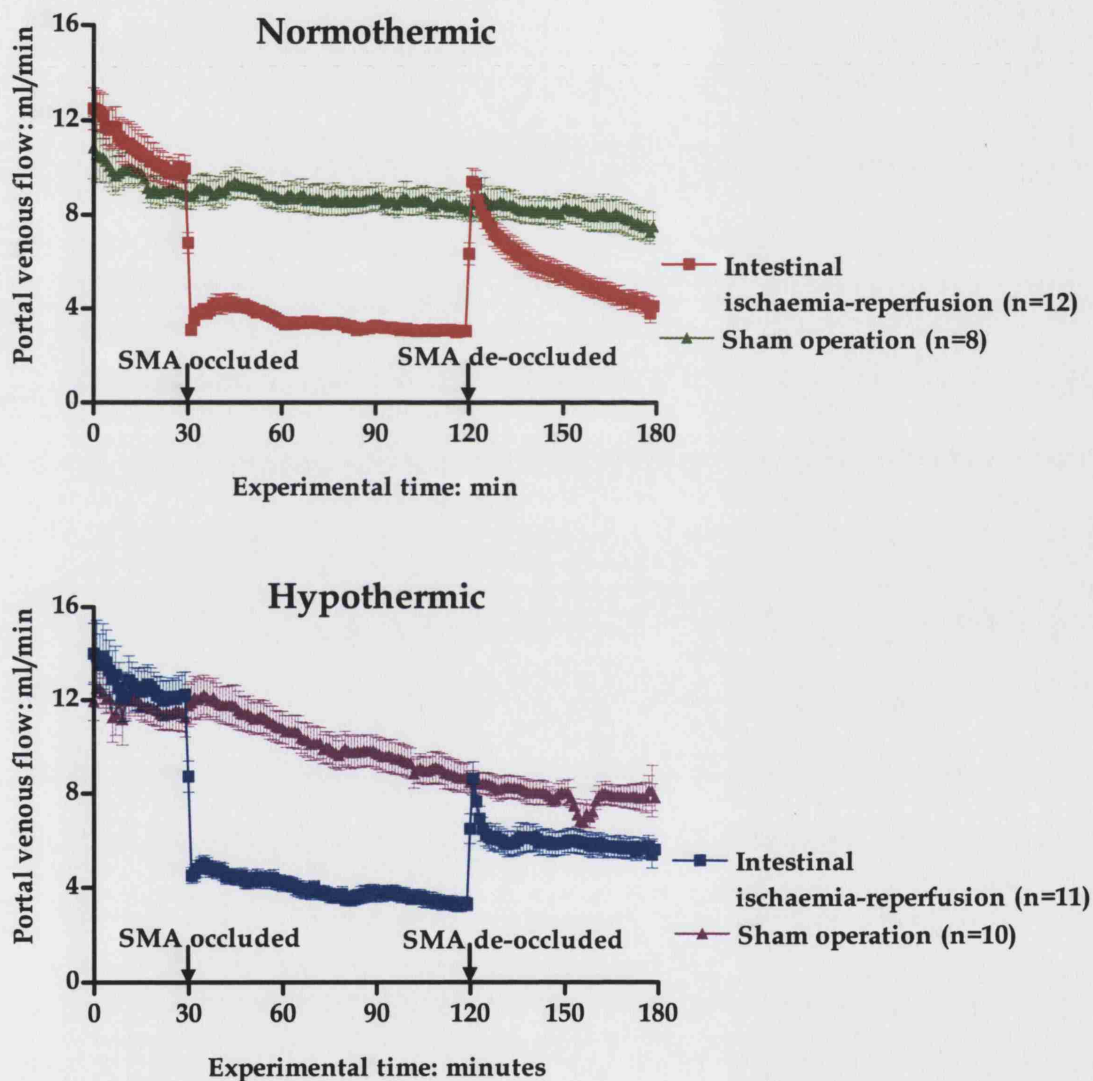


Figure 4.8: PVF during IIR and sham operation in normothermic and hypothermic rats: group data, 60-second group mean \pm SEM

Overall, a substantial decline in PVF with prolonged sham operation was seen at both normothermia and hypothermia, but was more pronounced at hypothermia. IIR resulted in a rapid and substantial fall in PVF immediately after

SMA occlusion that was sustained throughout intestinal ischaemia. SMA de-occlusion was followed by the rapid restoration of PVF to levels equivalent to IIR rats immediately before SMA occlusion and to sham rats at the same time point, but this flow rate was not sustained in either temperature group. Normothermic IIR rats experienced a progressive decline in PVF (mean drop of $42.8 \pm 5.4\%$ between 130th and 180th minute; $P < 0.0001$) throughout intestinal reperfusion with final flow rates approaching those seen during intestinal ischaemia. In contrast, hypothermic IIR rats showed a levelling off of PVF and sustained a moderately decreased PVF throughout the latter part of intestinal reperfusion at a level greater than that seen during intestinal ischaemia and greater than that of normothermic IIR rats.

Comparison of summary measurement points for PVF within and between treatment groups is shown in figure 4.9.

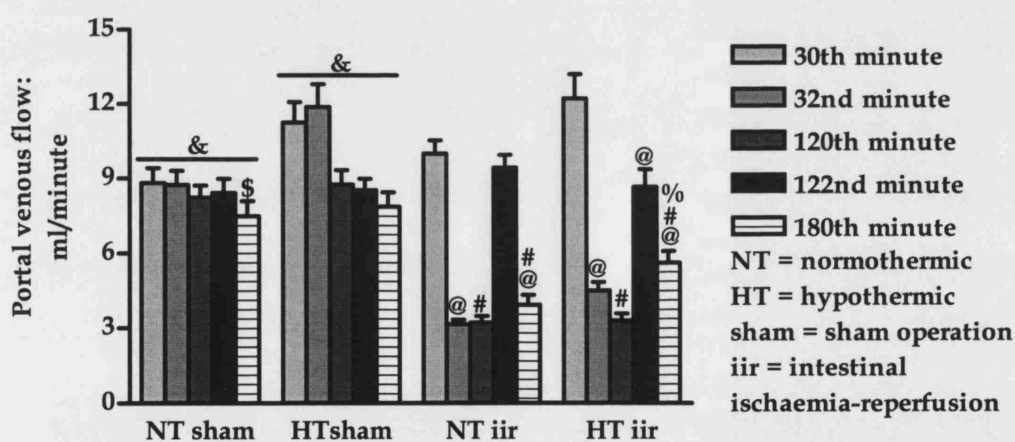


Figure 4.9: Summary measurements of PVF during IIR and sham operation in normothermic and hypothermic rats.

@ $P < 0.05$ versus 30th minute, same treatment

% $P < 0.05$ versus 120th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus NT iir, same time point

& $P < 0.05$ (change over experimental time)

A significant decline in PVF over experimental time was seen in both sham operation groups, and the decline was more marked in the hypothermic group, largely due to higher initial PVF. Normothermic and hypothermic IIR rats showed a large fall in PVF at the 32nd minute compared to the 30th minute – i.e. with SMA occlusion. Reduced PVF was sustained at the 120th minute in both temperature groups. In the normothermic IIR group, PVF equivalent to that in the 30th minute was achieved in the 122nd minute, but this was not seen in the hypothermic IIR rats, which showed an increase in flow between the 120th and 122nd minute – i.e. on reperfusion - but failed to reach pre-occlusion flow rates. After prolonged reperfusion PVF declined in both IIR temperature groups, but the fall was greater in the normothermic IIR group, becoming significantly lower than normothermic sham operation at the 180th minute and no different from PVF at the end of intestinal ischaemia. In the hypothermic group, PVF was maintained at the 180th minute at a rate greater than at the end of intestinal ischaemia and equivalent to hypothermic sham operation.

HAF group data (60 second group mean \pm SEM over experimental time) during IIR or sham operation at normothermia and hypothermia is shown in figure 4.10.

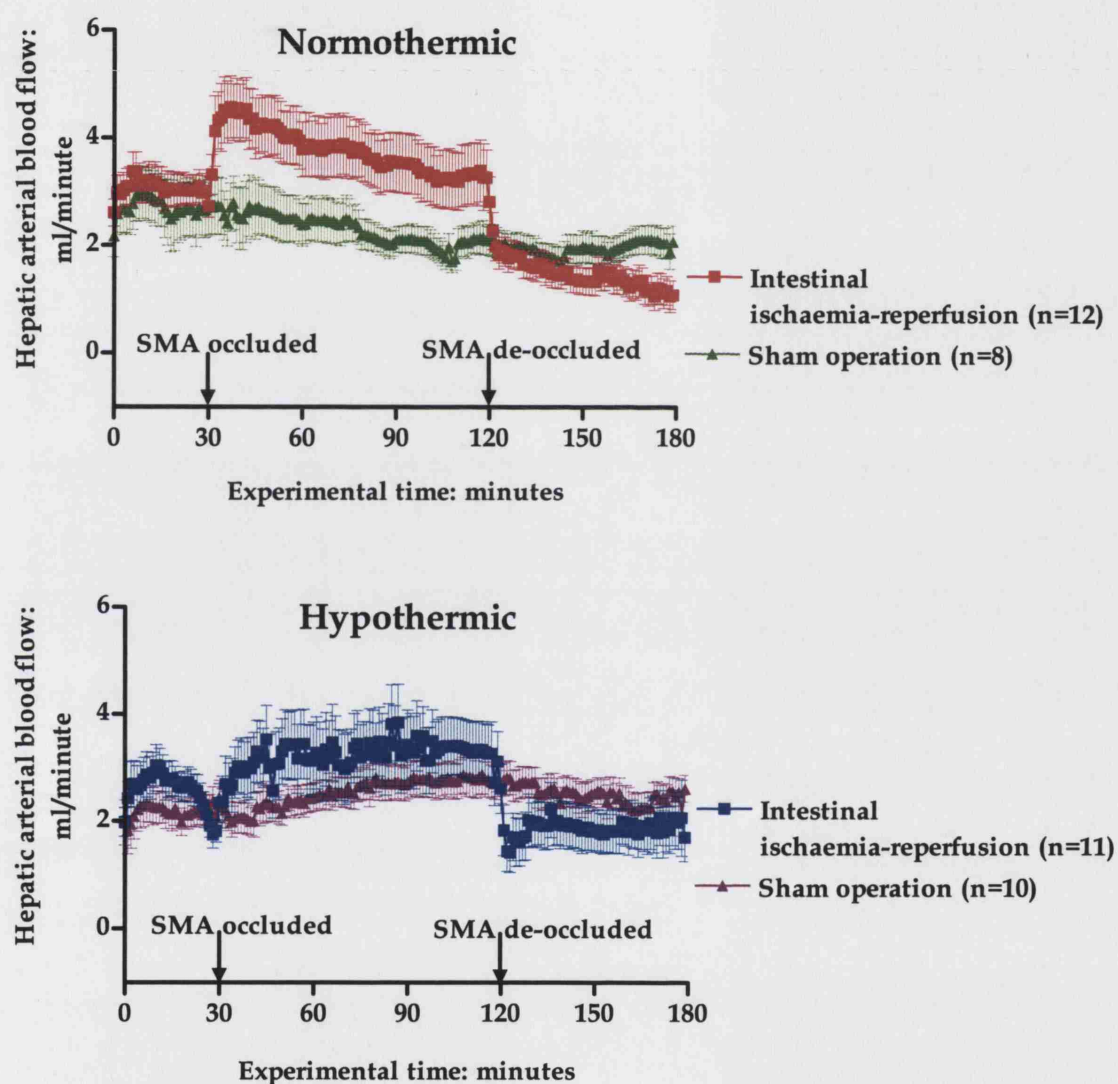


Figure 4.10: HAF during IIR and sham operation in normothermic and hypothermic rats: group 60-second mean \pm SEM

The 'hepatic arterial buffer response' (increase in HAF after a sustained decrease in PVF) is clearly seen in the IIR groups during intestinal ischaemia, but the increase in HAF in the normothermic group is not sustained throughout

intestinal ischaemia. HAF does not change significantly over experimental time in either sham group. Normothermic IIR results in a progressive decline in HAF during intestinal reperfusion (mean drop of $34.2 \pm 10.8\%$ between 130th and 180th minute; $P = 0.0090$) – a response that is not seen in the hypothermic IIR group. Final hepatic arterial flow is less than pre-IIR flow in the normothermic IIR group, but is unchanged in the hypothermic IIR group.

Comparison of summary measurement points for HAF within and between treatment groups is shown in figure 4.11.

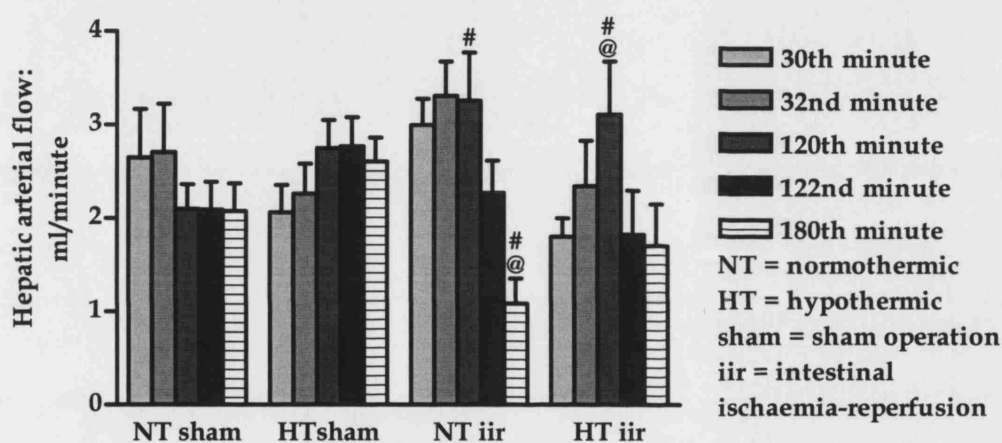


Figure 4.11: Summary measurements of HAF during IIR and sham operation in normothermic and hypothermic rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

No significant changes were seen in either sham operation group. A significant drop in HAF between the 120th and 122nd minute was seen in both IIR groups, returning HAF to a level equivalent to the 30th minute. In the normothermic IIR group, HAF continues to fall and by the 180th minute is significantly lower than in both the 30th and 122nd minutes, but not significantly lower than after normothermic sham operation. In the hypothermic IIR group, a peak in HAF is seen at the 120th minute with flow higher than at the 30th and 122nd

minutes. Final flow in the hypothermic IIR group is equivalent to the 30th minute and to flow at the 180th minute after hypothermic sham operation.

THI [PVF + HAF] group data (60 second group mean \pm SEM over experimental time) during IIR or sham operation at normothermia and hypothermia is shown in figure 4.12.

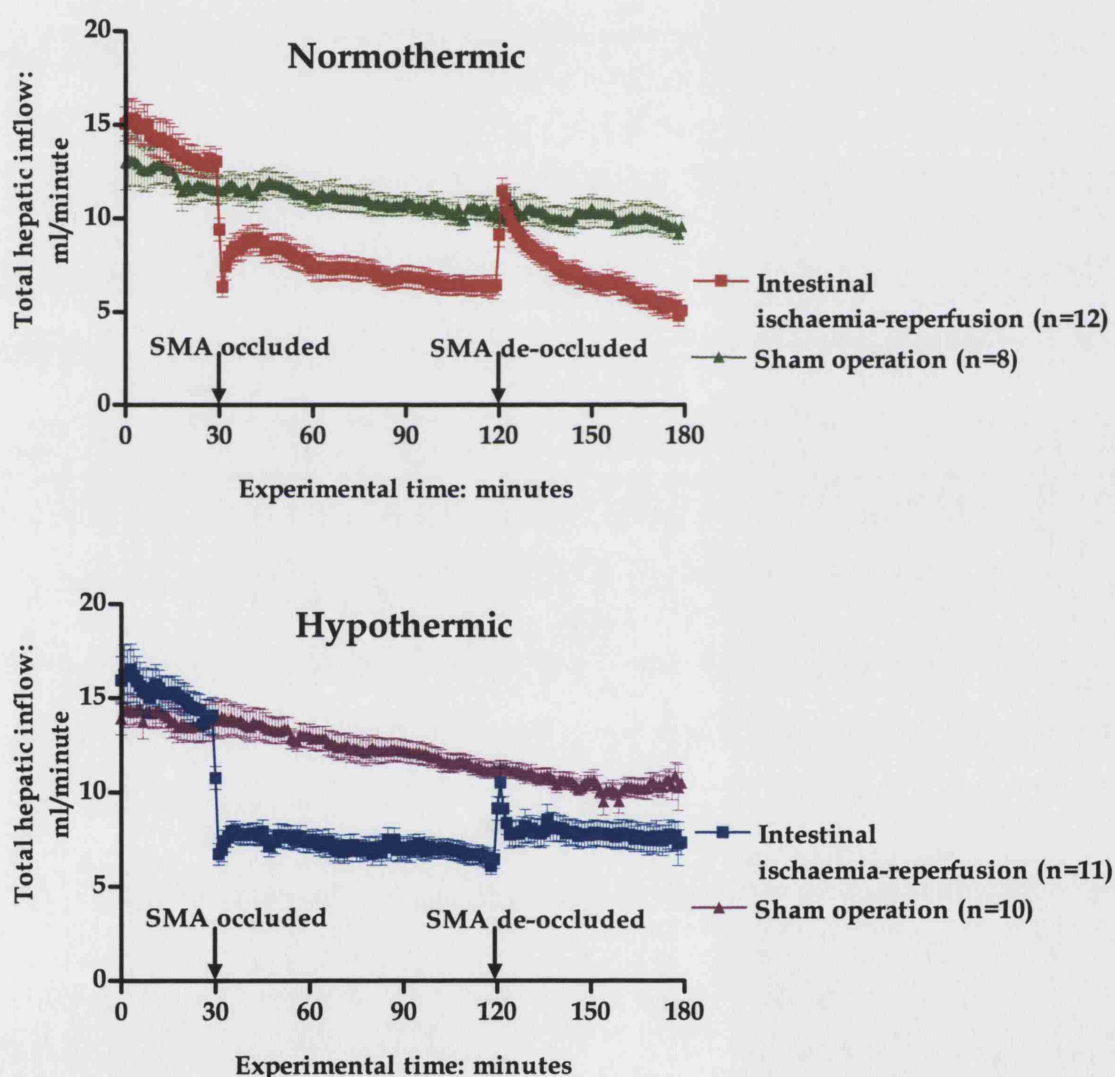


Figure 4.12: THI during IIR and sham operation in normothermic and hypothermic rats: group 60-second mean \pm SEM

Overall, a decline in THI is seen during sham operation at both temperatures – this is more marked in the hypothermic group. Normothermic IIR

rats showed a drop in THI with SMA occlusion and recovery of THI with SMA de-occlusion to reach flow equal to pre-occlusion levels and normothermic sham operation levels at the same time point. A progressive decline in THI was seen during normothermic intestinal reperfusion (mean drop of $41.4 \pm 6.0\%$ between 130th and 180th minute; $P < 0.0001$) with final flow rates at or below those at the end of intestinal ischaemia. Hypothermic IIR rats also showed a large drop in THI with SMA occlusion, but failed to achieve pre-occlusion flow rates on SMA de-occlusion, although flow was equal to that seen during hypothermic sham operation at the same time point. Unlike normothermic IIR rats during intestinal reperfusion, hypothermic IIR rats did not show a progressive decline in THI. THI levelled off rapidly (by 10 minutes of reperfusion) on reperfusion and was sustained without further change until the end of the experiment.

Comparison of summary measurement points for THI within and between treatment groups is shown in figure 4.13.

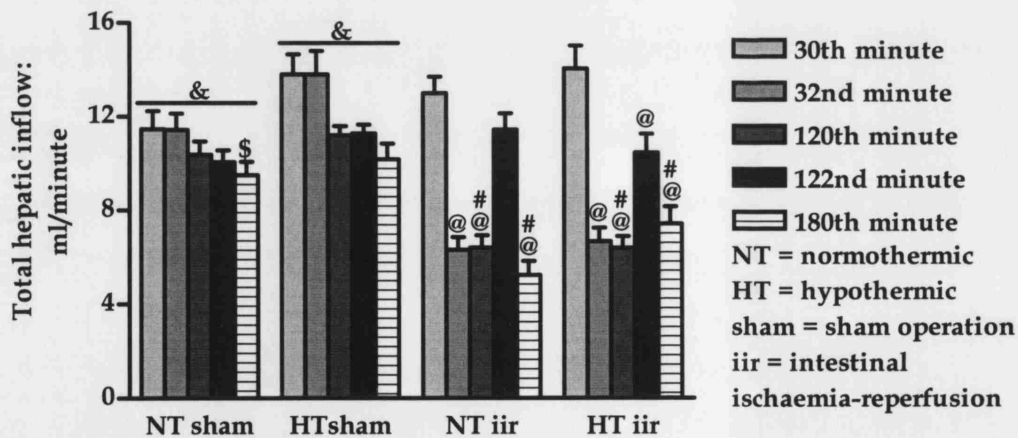


Figure 4.13: Summary measurements of THI during IIR and sham operation in normothermic and hypothermic rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus NT iir, same time point

& $P < 0.05$ (change over experimental time)

A significant decline in THI over experimental time was seen in both sham operation groups. Normothermic IIR rats showed a significant fall in THI between the 30th and 32nd minute which was sustained at the 120th minute. THI recovered to equivalence with the 30th minute by the 122nd minute in the normothermic IIR group, then declined to equal the 120th minute flow rate in the 180th minute, significantly lower than the flow rates seen in the 30th and 122nd minutes and in the 180th minute in the normothermic sham operation group. In the hypothermic IIR group a similar pattern was seen except that 122nd minute flow remained less than 30th minute flow (but was equivalent to 122nd minute flow in the hypothermic sham operation group) and 180th minute flow did not decline to a level significantly below that of the hypothermic sham operation group at the 180th minute.

Haematocrit, pH and blood oxygen content measurements

All data in this section describe samples taken at the end of the experiment (i.e. after 30 minutes run-in followed by 90 minutes intestinal ischaemia and 60 minutes intestinal reperfusion or 150 minutes sham operation).

Haematocrit of samples taken from the SMV, aorta and IVC after IIR or sham operation at normothermia and hypothermia is shown in figure 4.14.

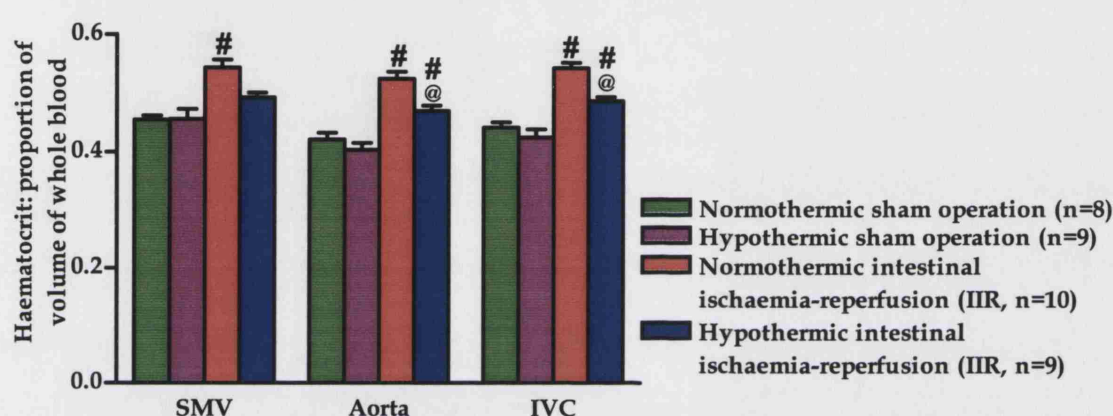


Figure 4.14: Haematocrit after IIR or sham operation in normothermic and hypothermic rats

$P < 0.05$ versus sham operation, same temperature and sample site

@ $P < 0.05$ versus normothermic group, same treatment and sample site

$P < 0.05$ (smv > ivc > aorta) for all experimental groups

Interaction between treatment and sample site: $P = 0.7848$

The pattern of changes in haematocrit seen in each sample site was the same for all treatment groups, with consistently higher haematocrit in the SMV than in the IVC or in the aorta for all treatments. Haematocrit rose after normothermic IIR at all sites compared to sham operation. Haematocrit rose after hypothermic IIR compared to sham operation only in the aorta and IVC, and these increases resulted in significantly lower haematocrit at these sites than were seen after normothermic IIR.

pH of samples taken from the SMV, aorta and IVC after IIR or sham operation at normothermia and hypothermia is shown in figure 4.15.

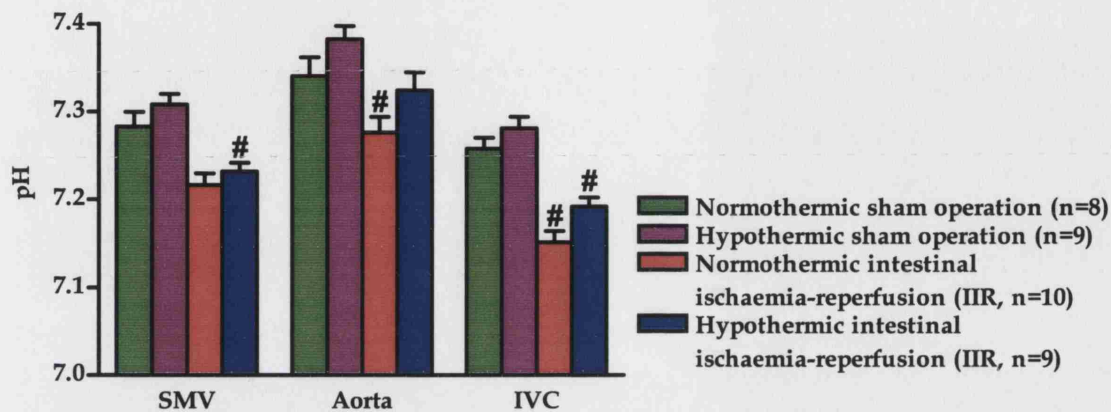


Figure 4.15: pH after sham operation or intestinal ischaemia-reperfusion in normothermic and hypothermic rats

$P < 0.05$ versus sham operation, same temperature and sample site

Interaction between treatment and site: $P = 0.5446$

The pattern of changes in pH seen in each sample site was the same for all treatment groups. pH of the aortic sample was consistently significantly higher than the pH of the IVC sample across all treatment groups, and at hypothermia only pH of the SMV sample was significantly lower than the pH of the aortic sample. Normothermic IIR resulted in lower pH in aortic and IVC samples than after normothermic sham operation, but not in SMV samples. Hypothermic IIR resulted in lower pH in SMV and IVC samples compared to hypothermic sham operation, but no change was noted in aortic samples.

Whole blood oxygen content of samples taken from the SMV, aorta and IVC after IIR and sham operation at normothermia and hypothermia is shown in figure 4.16.

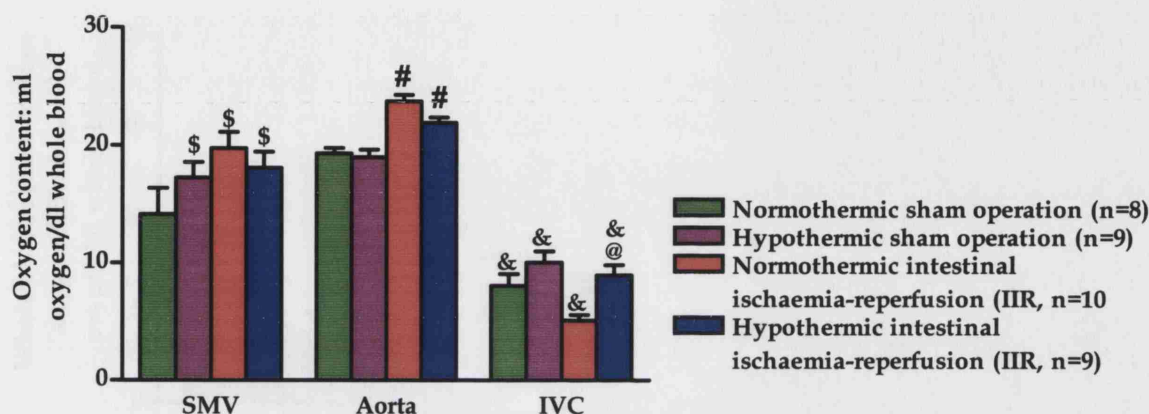


Figure 4.16: Whole blood oxygen content after IIR or sham operation in normothermic and hypothermic rats

$P < 0.05$ versus sham operation, same temperature

@ $P < 0.05$ versus normothermic group, same treatment and sample site

& $P < 0.05$ versus aorta, same treatment and temperature

\$ $P < 0.05$ versus inferior vena cava, same treatment and temperature

Interaction between treatment and sample site: $P = 0.0001$

The pattern of change seen in each sample site was not the same for all treatment groups: SMV oxygen content did not change with different treatments; aortic oxygen content rose with IIR compared to sham operation at both temperatures; IVC oxygen content fell with normothermic IIR compared to hypothermic IIR only. All treatments resulted in blood oxygen contents that were not significantly different between SMV and aortic samples, and higher in SMV than in IVC samples (except normothermic sham operation, after which no significant difference was found between SMV and IVC oxygen content). IIR resulted in higher aortic blood oxygen content compared to sham operation at both temperatures. Hypothermic IIR IVC blood oxygen content was greater than after normothermic IIR.

Whole blood A-V difference in oxygen content across the territories drained by the SMV and IVC after IIR or sham operation at normothermia and hypothermia is shown in figure 4.17.

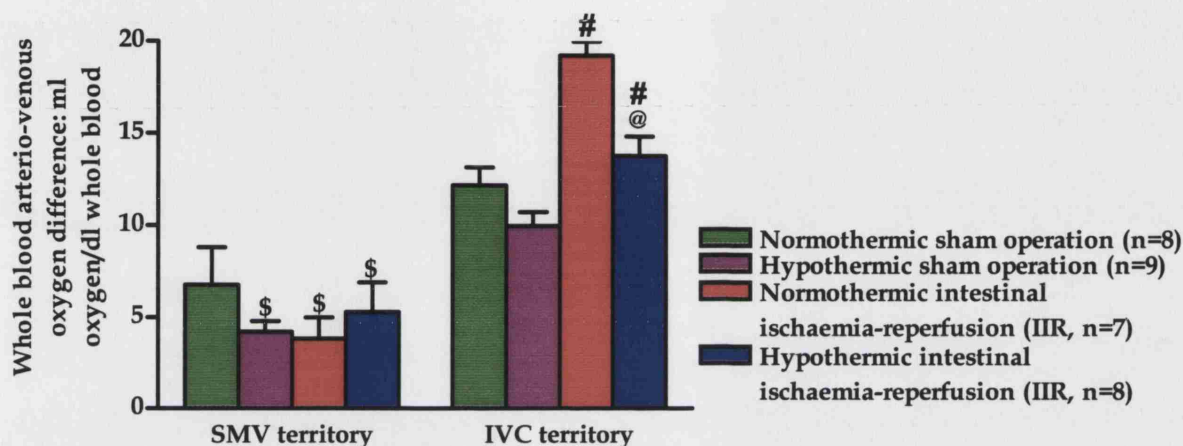


Figure 4.17: Whole blood A-V difference in oxygen content after IIR or sham operation in normothermic and hypothermic rats

$P < 0.05$ versus sham operation, same temperature

@ $P < 0.05$ versus normothermic group, same treatment and sample site

\$ $P < 0.05$ versus inferior vena cava, same treatment and temperature

Interaction between treatment and drainage territory: $P = 0.0002$

The pattern of change seen in each territory is not the same for different treatment groups. For all treatments except normothermic sham operation, whole blood A-V difference in oxygen content in territory drained by the IVC was higher than that drained by the SMV. No significant difference in whole blood A-V difference in oxygen content in territory drained by the SMV was seen after any treatment. In territory drained by the IVC, A-V difference in whole blood oxygen content was higher after IIR than after sham operation at either temperature. The rise in A-V difference in whole blood oxygen content after IIR in territory drained by the IVC was greater after normothermic IIR than after hypothermic IIR.

Liver metabolite measurement and correlation with final minute total hepatic inflow

A panel of hepatic metabolites (glucose, succinate, lactate, β -hydroxybutyrate, alanine, glutamate, glutamine, adenosine diphosphate [ADP], adenosine triphosphate [ATP], inorganic phosphate [Pi], glutathione) were measured after IIR or sham operation at normothermia and hypothermia (i.e. after 30 minutes run-in followed by either 90 minutes of intestinal ischaemia and 60 minutes of intestinal reperfusion or 150 minutes of sham).

No significant changes in hepatic concentration of glucose, succinate, lactate, β -hydroxybutyrate, glutamate, glutamine, ADP, Pi or glutathione were recorded in this experiment. This is in agreement with the results reported for adult rats after very similar insults in Chapter 2 (pages 121 – 131), with the exception of glutamine and Pi. For the metabolites that did show significant changes with IIR at normothermia or hypothermia – ATP and alanine – the impact of THI in the final minute of the experiment (i.e. immediately prior to tissue sampling) was explored by plotting THI in the final minute of the experiment against the hepatic tissue concentration of ATP and alanine.

Liver tissue ATP concentration after intestinal ischaemia-reperfusion and sham operation at normothermia or hypothermia is shown in figure 4.18.

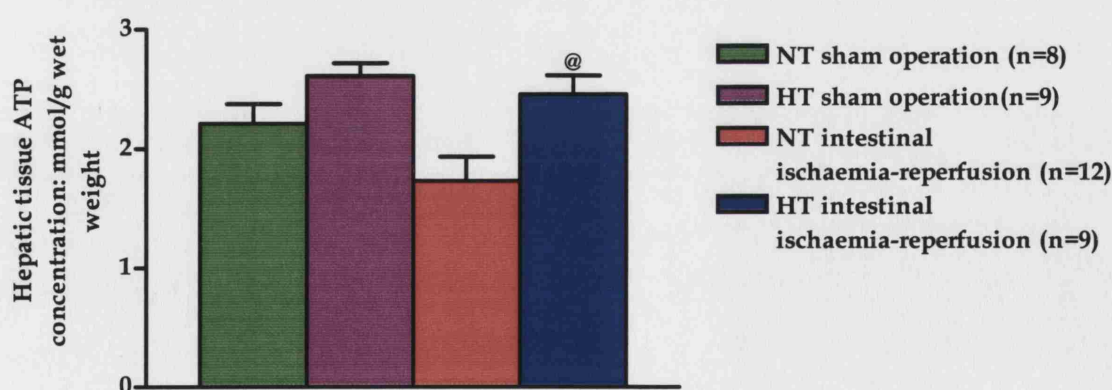


Figure 4.18: Hepatic tissue ATP concentration after IIR or sham operation at normothermia and hypothermia

@ $P < 0.05$ versus normothermic IIR group

Hepatic tissue ATP level plotted against total hepatic inflow in the final minute of the experiment for each experimental animal is shown in figure 4.19.

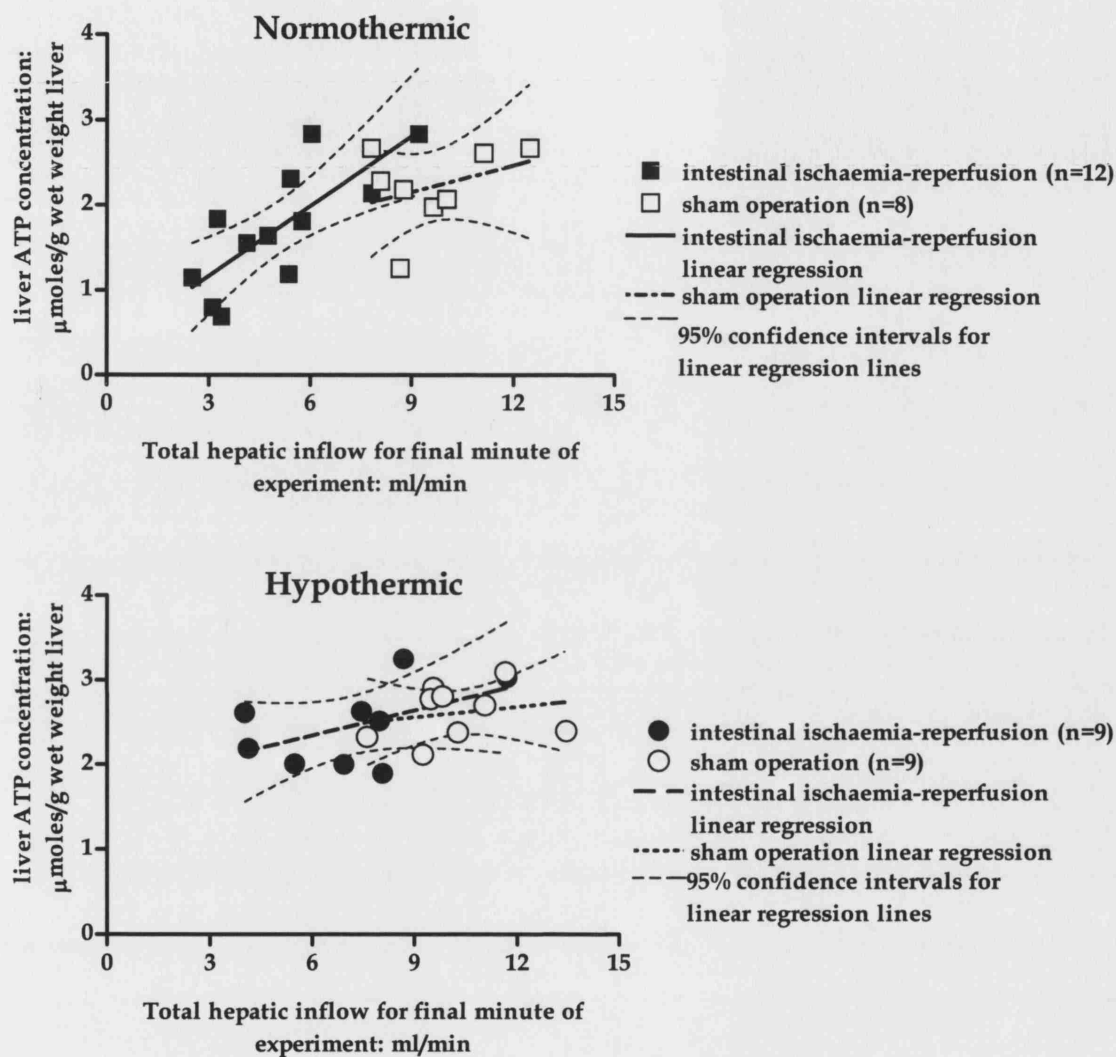


Figure 4.19: Liver ATP concentration *versus* total hepatic inflow for the final minute of the experiment after intestinal ischaemia-reperfusion or sham operation in normothermic and hypothermic rats

Linear regression:

P (slope \neq zero) < 0.05 normothermic intestinal ischaemia-reperfusion

P (slope \neq zero) > 0.05 all other experimental groups

$P > 0.05$ (slopes equivalent)

The only group to show a significant correlation between hepatic tissue ATP level and total hepatic inflow immediately prior to tissue sampling was normothermic IIR – this group showed a positive correlation between hepatic tissue ATP and final hepatic inflow.

Liver tissue alanine concentration after intestinal IIR or sham operation at normothermia or hypothermia is shown in figure 4.20.

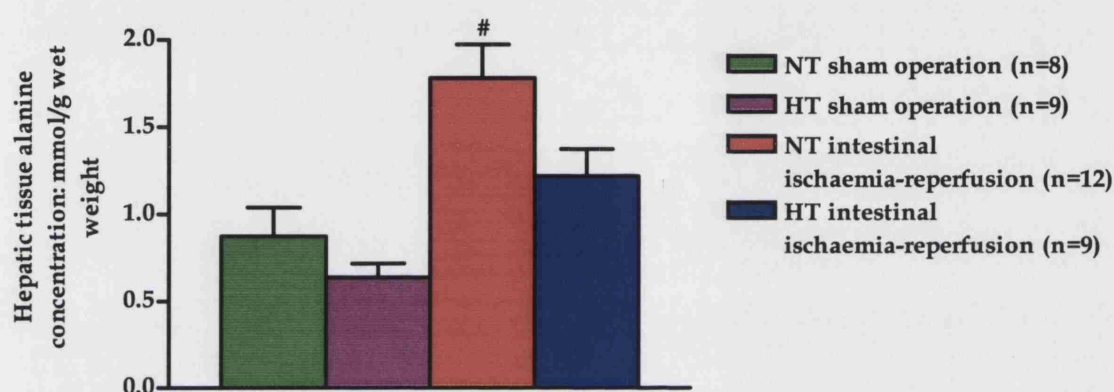


Figure 4.20: Hepatic tissue alanine concentration after intestinal ischaemia-reperfusion and sham operation at normothermia and hypothermia

$P < 0.05$ versus normothermic sham operation group

Hepatic alanine levels were significantly higher after normothermic IIR than after normothermic sham operation. This rise was not seen in the hypothermic IIR group.

Hepatic tissue alanine level plotted against total hepatic inflow in the final minute of the experiment for each experimental animal is shown in figure 4.21.

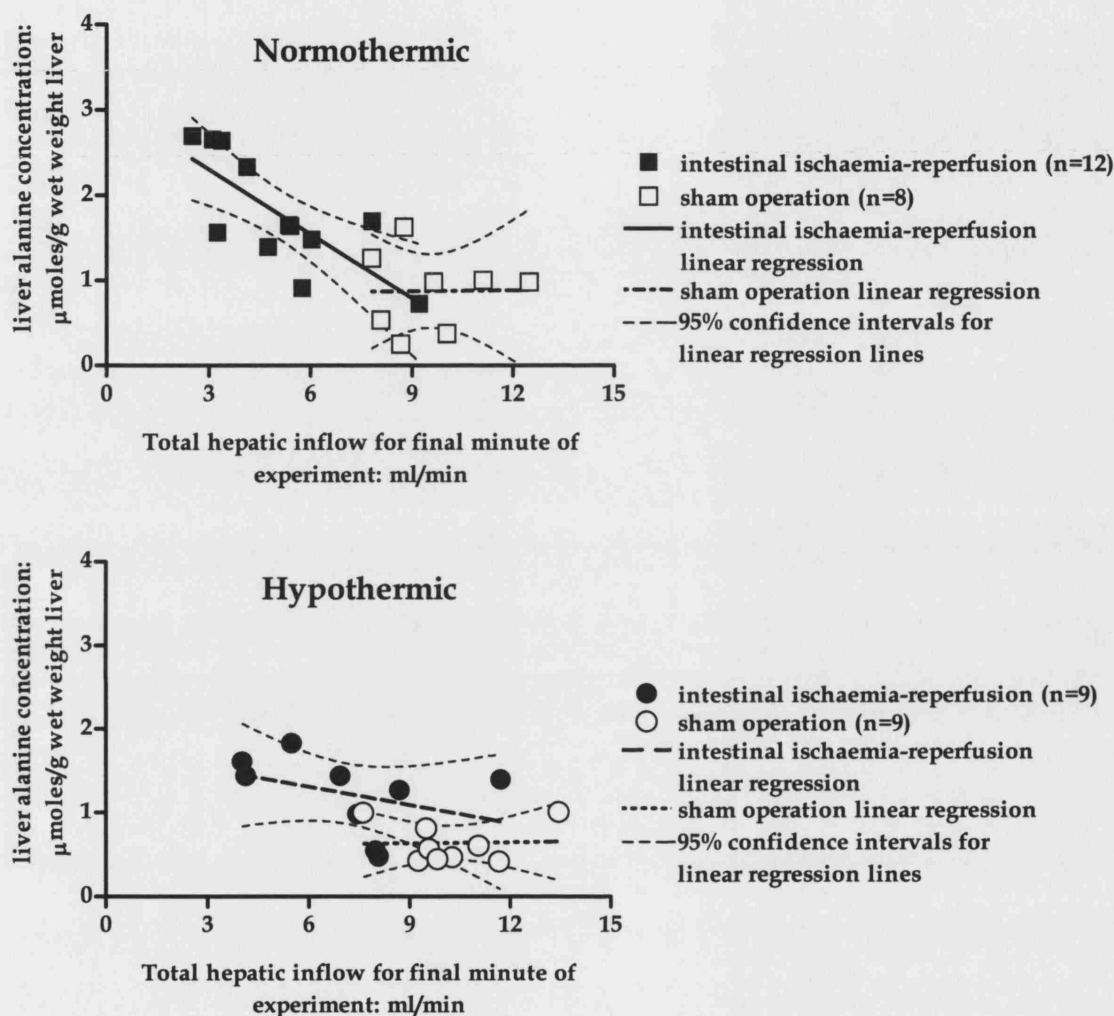


Figure 4.21: Liver alanine concentration *versus* total hepatic inflow for the final minute of the experiment after IIR or sham operation in normothermic and hypothermic rats

Linear regression:

P (slope \neq zero) < 0.05 normothermic intestinal ischaemia-reperfusion

P (slope \neq zero) > 0.05 all other experimental groups

$P > 0.05$ (slopes equivalent)

The only group to show a significant correlation between hepatic tissue alanine level and total hepatic inflow immediately prior to tissue sampling was normothermic IIR – this group showed a negative correlation between hepatic tissue alanine and final hepatic inflow.

Discussion

This experiment demonstrates real-time monitoring of heart rate, arterial blood pressure and hepatic inflow during IIR and sham operation in a rat model at normothermia and hypothermia. This model has also been used to analyse some of the effects of whole body hypothermia on remote organ injury after IIR by demonstrating effects on haematocrit, pH, whole blood oxygen content and A-V difference across SMV and IVC capillary beds, and hepatic metabolites.

The sustained mean rise in heart rate during IIR at normothermia was expected as IIR is known to induce circulatory collapse (Hayward and Lefer, 1999) and an increase in heart rate with prolonged reperfusion (Khanna et al, 2001). The significant fall in heart rate during intestinal reperfusion with hypothermia was unexpected, and demonstrates a profoundly different reaction to intestinal reperfusion to that seen at normothermia. A fall in heart rate with whole-body cold stress (to 32°C) has been reported in young pigs (Powell et al, 1999). This was associated with reductions in both cerebral and intestinal blood flow and a fall in cardiac output. Cardiac output and intestinal inflow remained depressed following rewarming, indicating a profound and lasting effect of hypothermia on cardiac function and the mesenteric vascular bed. Dae et al (Dae et al, 2002) used endovascular cooling to lower core body temperature to 34°C in large pigs and demonstrated a reduction in myocardial infarct size after occlusion of the left anterior descending artery. This was accompanied by a significant reduction in heart rate in hypothermic pigs, who maintained cardiac output by increasing stroke volume. Hypothermia may therefore have a direct effect on cardiac

pacemaker tissue when under physiological stress (i.e. after myocardial infarction or IIR), slowing heart rate but allowing stroke volume to increase thus efficiently maintaining cardiac output.

The small but significant increase in MAP over experimental time during normothermic sham operation was not accompanied by a progressive change in heart rate, and may be due to prolonged halothane anaesthesia in the absence of any other stimulus. Hypothermic sham operation rats showed no variation in either heart rate or MAP despite identical halothane exposure, suggesting that whole-body hypothermia may prevent this postulated effect of prolonged halothane exposure without further stimulus. Both normothermic and hypothermic IIR rats demonstrated the widely reported drop in MAP immediately on intestinal reperfusion (Nakamura et al, 2001; Schmeling et al, 1989). It is interesting to note that hypothermic IIR animals showed a deeper and longer fall in MAP on intestinal reperfusion than normothermic IIR animals. It has already been discussed that normothermic IIR animals responded to prolonged intestinal reperfusion with an increased heart rate that maintained MAP until the end of the experiment. Hypothermic IIR animals responded to prolonged intestinal reperfusion with a reduction in heart rate, which may have prolonged the fall in MAP observed immediately after reperfusion. The ability of this group to maintain MAP until the end of the experiment whilst relatively bradycardic is surprising. Possible explanations for this may be the global reduction in effects of IIR at hypothermia shown in the remainder of this experiment. Smaller reductions in portal and total hepatic inflow compared to sham operation at the end of the experiment could indicate less hepatic hypoperfusion with prolonged intestinal reperfusion when hypothermic. Reduced haemoconcentration with hypothermia may indicate less relative hypovolaemia secondary to endovascular leak after IIR at hypothermia. Maintenance of hepatic tissue ATP and alanine concentrations could indicate less remote organ damage after IIR at hypothermia. In the experiment reported in Chapter 5 of this thesis (pages 227 - 262), cytokine and

endotoxin release after IIR are significantly attenuated at hypothermia, indicating a reduction in the local and systemic inflammatory response to IIR and reduced endotoxin escape after IIR.

In support of the hypothesis leading this experiment, IIR and sham operation had substantial effects on blood flow into the liver. A significant sham operation effect was seen at both normothermia and hypothermia, with a fall in PVF over experimental time at both temperatures that was not compensated for by an increase in HAF and was therefore reflected in a fall in THI over experimental time. Other groups have reported large sham operation effects when examining the effect of IIR on hepatic inflow: 60% and 50% reductions in portal venous and hepatic arterial flow respectively after 180 minutes of sham operation under general anaesthetic in rats (Turnage et al, 1996); a gradual decrease in PVF with sham operation only under general anaesthetic in dogs after 6 hours (Nakamura et al, 2001). The mechanism behind these falls in PVF with prolonged general anaesthetic after minimal surgical intervention is not clear, but may be due to a gradual diversion of blood flow away from the splanchnic circulation during the minor physiological stress of general anaesthesia and laparotomy. This mirrors the findings of Anup et al (Anup et al, 1999) who described subtle intestinal changes with an insult similar to sham operation under general anaesthetic in rats.

Normothermic IIR resulted in the expected pattern of a large and sustained drop in PVF with SMA occlusion, transient restoration of PVF to run-in levels on SMA de-occlusion, and a gradual diminution of PVF with prolonged intestinal reperfusion to a level significantly below that of the normothermic sham operation at the end of the experiment. The 'hepatic arterial buffer response' was not demonstrated during intestinal reperfusion, and a significant reduction in HAF (despite low portal venous flow) was seen in the final minute of the experiment. These changes were reflected in THI with a steady decline recorded over the last 50 minutes of the experiment. Similar patterns have been reported after IIR at normothermia by other groups (Nakamura et al, 2001; Turnage et al, 1996). The

resistance of hypothermic animals to the effects of IIR is seen in the changes in pattern of flow in the portal vein and hepatic artery during intestinal reperfusion. Final PVF in the hypothermic IIR group was not significantly lower than that of the hypothermic sham operation group, demonstrating a remarkable sustenance of PVF despite a severe intestinal insult. The 'hepatic arterial buffer response' was apparent in the hypothermic IIR group by the end of intestinal ischaemia, indicating the preservation of normal hepatic control of dual blood flow at hypothermia only, and HAF did not decline in this group with prolonged intestinal reperfusion. The overall effect of hypothermia on IIR was to ameliorate the effects of IIR on hepatic inflow and resulted in the sustenance of THI at the end of the experiment at levels not significantly lower than those seen after sham operation at hypothermia. The mechanisms behind this effect are not clarified by this experiment, but it may be speculated that prevention of the fall in intestinal outflow demonstrated after IIR by Schmeling et al (Schmeling et al, 1989) and Turnage et al (Turnage et al, 1995) and/or a reduction in the increase in mesenteric vascular resistance demonstrated after IIR by Nankervis et al (Nankervis et al, 2000) by whole body hypothermia may be involved. This effect should be considered in the context of the blood composition data also presented here – a reduction in the increase in haematocrit seen after normothermic IIR after whole body hypothermia IIR - that may further explain this contrast. IIR at normothermia is associated with an increase in vascular permeability (Sun et al, 2002; Ward et al, 2000); an effect that may be dependent on platelet-activating factor [PAF] (Noel et al, 1996). Hypothermia may have reduced the loss of plasma into interstitial tissue within the intestine and thus preserved blood volume, maintaining flow from the intestine into the liver. Prevention of the fall in hepatic arterial inflow after 60 minutes of intestinal reperfusion at hypothermia was important in maintaining THI and suggests that hypothermia ameliorated the global effects of IIR. An explanation for this may be seen in the data presented in Chapter 5 of this thesis (pages 227 - 262) – if hypothermia reduces the overall

inflammatory impact of IIR, the global effects of IIR (i.e. on hepatic arterial inflow after IIR) may be prevented.

Partial protection is provided against haemoconcentration, but not against a fall in blood pH, by hypothermic IIR. The mechanism behind the effect on haematocrit is possibly a reduction in capillary permeability increase after IIR (Ward et al, 2000) resulting in less sequestration of plasma in the intestine and in the peripheral tissue on reperfusion. This may reflect a reduction in inflammatory response both locally and systemically, as reflected in samples taken from the portal and systemic circulations. The lack of beneficial effect on pH is harder to explain. It may reflect a more direct toxic effect of IIR on hydrogen ion homeostasis that is not alleviated by hypothermia. Vejchapipat et al (Vejchapipat et al, 2001) has shown that hypothermia does not reduce the adverse impact of IIR on intestinal metabolism, indicating that one source of hydrogen ions after IIR would be preserved after hypothermic IIR.

Increased whole blood oxygen content in the aorta also was not altered by hypothermia, although the reduction in oxygen content in the IVC was abrogated. This latter effect is likely to reflect the preservation of relatively normal transit times for blood in the systemic periphery after IIR with hypothermia, thus avoiding stagnation and increased peripheral oxygen extraction seen after IIR at normothermia in association with low MAP and tachycardia. This effect is marked, and is reflected in a reduction in oxygen extraction in the territory drained by the IVC after IIR at hypothermia compared to IIR at normothermia, although consumption remains greater than that seen after sham operation at hypothermia. As predicted by the work of Vejchapipat et al (Vejchapipat et al, 2001), no change was seen in oxygen extraction in the intestines after IIR at hypothermia reflecting the similarity in metabolic state of the intestines after IIR at both normothermia and hypothermia. The lack of impact of the relative reduction in blood flow through the intestines after normothermic IIR is not explained by these results. It

is possible that a degree of arterio-venous shunting may be present after IIR that is supported by the very low levels of oxygen consumption recorded here.

Measurements of liver tissue metabolites revealed a protective effect of hypothermia on hepatic ATP levels after IIR, and a reduction of the rise in hepatic tissue alanine after IIR with hypothermia. It is to be noted that the surgical preparation of the hepatic artery for this experiment is likely to have resulted in hepatic sympathectomy. Chronic hepatic sympathectomy in dogs has been shown to alter hepatic handling of a glucose load (Adkins-Marshall et al, 1992). It is possible therefore that the hepatic metabolic response to IIR in this experiment may have been affected by this. As the same surgical preparation was employed for all the experimental animals studied, it is unlikely that any inter-experimental group differences are due to this.

The changes in hepatic levels of alanine and ATP after IIR noted in this experiment are in agreement with Vejchapipat et al (Vejchapipat et al, 2001). In contrast to experiments reported in Chapter 2 of this thesis (pages 121 – 131), no significant changes were seen in glutamine or inorganic phosphate levels after IIR. It is not clear why these results do not agree – it may reflect the hepatic sympathectomy noted above or perhaps the prolonged nature of the experiments discussed in this chapter: it was clear that prolonged sham operation has significant effects on hepatic inflow and may also result in metabolic perturbation of the liver thus reducing the measured effects of IIR. Lengthy instrumentation of the hepatic inflow vessels may also have had an effect. Correlation of hepatic tissue metabolite concentration against final minute THI revealed a positive correlation between ATP and final minute THI, and a negative correlation between hepatic alanine and final minute THI after normothermic IIR only. This suggests that low flow rates into the liver after IIR either directly or indirectly reflect a process that also affects hepatic metabolism. The range of flow rates seen in the final minute of the experiment was similar in the normothermic and hypothermic IIR groups indicating that range bias is unlikely to have been important in this

analysis. The mechanism behind the increase in hepatic tissue alanine and decrease in hepatic tissue ATP is not clarified by this experiment, but may be related to the changes in cytokine and endotoxin concentrations after IIR described in Chapter 5 of this thesis (pages 227 - 262). It is possible to speculate that a critically low flow into the liver in a toxic environment may have resulted in the perturbation of metabolism in hepatocytes due to a combination of altered rate of oxygen delivery (it is noted that although the oxygen capacity of blood in the superior mesenteric vein is not significantly altered by IIR, the rate of delivery of blood to the liver is reduced after normothermic IIR by the end of the experiment), increase in blood viscosity (possibly causing sinusoidal stasis and increased transit time) and decrease in pH. In contrast, hypothermic IIR did not demonstrate a significant correlation between final total hepatic inflow and alanine or ATP. The similarity of ranges of final flow rates makes the lack of relationship with hepatic metabolite concentrations more important, as liver metabolism has apparently been preserved even in the presence of very low hepatic inflow in the hypothermic IIR group.

Summary and Conclusions

The effect of IIR and sham operation at normothermia and hypothermia on heart rate, MAP and hepatic inflow were followed in real time in a rat model using a transduced intra-arterial catheter and transit-time ultrasound flow probes.

Sham operation was associated with a progressive rise in MAP without a progressive change in heart rate at normothermia only, and a progressive fall in PVF at both temperatures that was reflected in THI.

Normothermic IIR was associated with a rise in heart rate without a significant change in MAP during intestinal reperfusion only; it was associated with a marked and sustained drop in PVF during SMA occlusion that recovered immediately on SMA de-occlusion, only to fall progressively throughout the remainder of intestinal reperfusion. The 'hepatic arterial buffer response' was not seen during intestinal reperfusion in this group, and HAF actually fell late in intestinal reperfusion. The overall effect on THI during intestinal reperfusion was a steady decline to a level less than that seen after normothermic sham operation at the end of the experiment. This was accompanied by an increase in haematocrit, fall in systemic pH (but no change in portal pH), and rise in A-V difference in whole blood oxygen content in the territory drained by the IVC. Hepatic tissue levels of ATP fell and alanine rose at the end of the experiment and correlated (positively and negatively respectively) with final minute THI.

In contrast, hypothermic IIR resulted in a drop in heart rate during intestinal reperfusion; maintenance of PVF after the first 10 minutes of intestinal reperfusion; preservation of the 'hepatic arterial buffer response'; and maintenance of HAF during intestinal reperfusion. The overall effect of IIR at hypothermia was to preserve final THI at hypothermic sham operation levels at the end of the experiment. Haemoconcentration was less marked; pH was unaltered in the aorta (but fell in the IVC and SMV); and whole blood oxygen content was preserved in

the IVC with a reduction in the increase in A-V difference in whole blood oxygen content in the territory drained by the IVC seen after normothermic IIR. Hepatic tissue ATP and alanine levels were preserved, and no correlation with final THI was found after hypothermic IIR.

The mechanisms behind the beneficial effects of hypothermia during IIR are not directly elucidated by this experiment but may involve reduction of vascular permeability changes, preservation of hepatic metabolism and vascular reactivity, and abrogation of the inflammatory response to IIR.

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Chapter 5: Whole body hypothermia attenuates cytokine and endotoxin responses to intestinal ischaemia-reperfusion

Introduction

In common with many other pathological insults (burns, haemorrhage, surgical trauma), intestinal ischaemia-reperfusion (IIR) results in the activation of an inflammatory cascade (Bitterman et al, 1991; Edelson, 1999; Syk et al, 1998; Willetts, 2001; Wyble et al, 1996). This is manifest by the release of pro- and anti-inflammatory cytokines including interleukin 6 (IL-6), interleukin 1- β (IL-1 β), tumour necrosis factor- α (TNF- α) and interleukin 10 (IL-10) (Bathe et al, 1998; Ding et al, 2001; Grotz et al, 1999; Hierholzer et al, 1999; Stallion, 2002; Weighardt, 2002). These inflammatory mediators are measurable locally, at the site of ischaemia-reperfusion injury, and systemically (Towfigh et al, 2000). Actions of cytokines released and time-courses of release depend on the nature of the insult received, the cytokine concerned, the concentration of cytokine achieved and the presence of other cytokines both in the immediate locality and circulating systemically (Hierholzer et al, 1999; Ledeboer, 2002; Stallion, 2002; Wyble et al, 1996). Cytokine release and action are also significantly influenced by the presence of other bioactive molecules – for example endotoxin (Ding et al, 2001; Grotz et al, 1999).

Because both pro-inflammatory (IL-1 β , IL-6 and TNF- α) and anti-inflammatory (IL-10) cytokines are released, a delicate balancing act is initiated aimed at providing an adequate response to the inflammatory trigger without producing either an overwhelming pro-inflammatory reaction or an exaggerated anti-inflammatory response (Cuzzocrea et al, 1999; Edelson, 1999; Mokart, 2002; Simonet et al, 1994; Suwa, 2002; Weighardt, 2002; Zingarelli et al, 2001). An uncontrolled pro-inflammatory response may cause more harm than the original

insult, and total suppression of inflammation may give rise to immune anergy and prevent an effective response to the original insult. A further complication is the presence of a normal cytokine response but a greater susceptibility to IIR or sepsis with certain genetic backgrounds (Godshall et al, 2002).

A complicating factor in ischaemia-reperfusion injury arising in the intestine is the presence of a large reservoir of endotoxin and endotoxin-bearing bacteria in the gut which may permeate or translocate through mucosa damaged by ischaemia-reperfusion injury and enter the portal circulation (Bathe et al, 1998; Ding et al, 2001; Drewe, 2001; Gathiram et al, 1989). Endotoxin could then be delivered to the liver in high concentrations, potentially triggering an inflammatory reaction remote to the site of original injury (Cohen et al, 1992; Koay, 2002). If endotoxin is not removed from plasma within the liver, it could go on to enter the systemic circulation (Cohen et al, 1992; Colletti et al, 1990; Towfigh et al, 2000). Endotoxin would then circulate systemically and be able to affect other organs remote to the site of original insult.

It is difficult to interfere with the propagation of this inflammatory cascade once it has been triggered. Evidence available at present from animal studies and clinical trials (Fijen et al, 2001; Majetschak, 1999) indicates that moderation of a single mediator from the cascade does not effectively reduce the sequelae of cascade activation (Koay, 2002; Stallion, 2002). A more global measure of cascade suppression was explored in this study: namely whole body hypothermia.

Whole body hypothermia has been shown to be protective of the biochemistry of the liver and is associated with reduced mortality after IIR (Vejchapipat et al, 2001). It has not been shown to be protective of the gut with regard to either morphology or biochemistry after intestinal ischaemia-reperfusion (Vejchapipat et al, 2000). The experiment detailed in Chapter 4 of this thesis (pages 173 – 220) has demonstrated some of the effects of whole body hypothermia (core temperature $32 \pm 0.5^{\circ}\text{C}$) during IIR on liver inflow, systemic haemodynamics,

hepatic metabolism and portal and systemic blood composition. In summary, whole body hypothermia resulted in blunting of the haemodynamic impact of IIR on the systemic circulation, a reduction in systemic haemoconcentration, a reduction in the rise in portal blood oxygen content, and a reduction in the rise in systemic arterio-venous difference in whole blood oxygen content after IIR. Whole body hypothermia also removed the positive correlation between final total hepatic inflow and liver adenosine triphosphate (ATP) concentration and negative correlation between final total hepatic inflow and liver alanine concentration.

The mechanism by which whole body hypothermia affords protection is not known. It is hypothesized here that the mechanism could involve moderation of cytokine and/or endotoxin release after IIR. This may be manifest via a global reduction in cytokine release, a differential increase in anti-inflammatory cytokine release, a reduction in endotoxin release or a combination of these effects.

The aim of this study was to establish the effect of intestinal reperfusion after intestinal ischaemia with and without whole body hypothermia on the concentration of selected pro- and anti-inflammatory cytokines (interleukins 1 β , 6 and 10 and tumour necrosis factor- α) and endotoxin in the portal and systemic circulations in a rat model. Samples were taken early in reperfusion (after 2 and 10 minutes), at the mid-point of reperfusion (after 30 minutes) and late in reperfusion (after 60 minutes). These time-points were chosen in order to provide a simple profile of changes in cytokine and endotoxin level during reperfusion. Time-matched samples were taken from sham operated animals only after 92, 120 and 150 minutes in order to minimise use of experimental animals.

Materials and Methods

Animal preparation

Age-matched male Sprague-Dawley rats (age 8 – 12 weeks; median weight 270g; weight range 234g - 418g) were studied. Rats were kept under standardised conditions for food, water, light and temperature. All animals had access to standard rat chow and water *ad libitum*.

There were 11 experimental groups:

A, B, C: Normothermic sham operation for 92 min (n = 6), 120 min (n = 6), or 150 min (n = 11)

D, E, F, G: Normothermic intestinal ischaemia-reperfusion (90 minutes ischaemia + X minutes reperfusion): X = 2 (n = 8), X = 10 (n = 9), X = 30 (n = 9) or X = 60 (n = 12).

H, I, J, K: Hypothermic intestinal ischaemia-reperfusion (90 minutes ischaemia + X minutes reperfusion): X = 2 (n = 8), X = 10 (n = 7), X = 30 (n = 7) or X = 60 (n = 7).

Chapter 5: Whole body hypothermia attenuates cytokine and endotoxin responses to intestinal ischaemia-reperfusion

Summary table:

Group	Temperature	Treatment	Duration of experiment	Number of animals
A	Normothermic	Sham	92 min	6
B	Normothermic	Sham	120 min	6
C	Normothermic	Sham	150 min	11
D	Normothermic	IIR	90 min ii + 2 min ir	8
E	Normothermic	IIR	90 min ii + 10 min ir	9
F	Normothermic	IIR	90 min ii + 30 min ir	9
G	Normothermic	IIR	90 min ii + 60 min ir	12
H	Hypothermic	IIR	90 min ii + 2 min ir	8
I	Hypothermic	IIR	90 min ii + 10 min ir	7
J	Hypothermic	IIR	90 min ii + 30 min ir	7
K	Hypothermic	IIR	90 min ii + 60 min ir	7

ii = intestinal ischaemia by superior mesenteric artery occlusion
ir = intestinal reperfusion

A hypothermic sham group was considered but rejected in line with government policy on the use of animals in research ('Replace, Reduce, Refine').

Rectal temperature was monitored continuously immediately after induction of deep surgical anaesthesia (absence of response to firm web-space pinch). Hypothermic animals were allowed to cool passively under general anaesthetic to $32 \pm 0.5^{\circ}\text{C}$ prior to the onset of the experiment. Animals were maintained at $37 \pm 0.5^{\circ}\text{C}$ for the normothermic groups and $32 \pm 0.5^{\circ}\text{C}$ for the hypothermic groups using a heating pad and radiant lamp.

Surgical procedure

All animals were anaesthetised with 3% halothane by inhalation in a 50:50 mixture of oxygen and nitrous oxide at a flow rate of 200ml/min per gas, until deep surgical anaesthesia had been induced. Anaesthesia was maintained using 0.8-1.5% halothane in a 50:50 mixture of oxygen and nitrous oxide at the same flow rates for the remainder of the experiment. All animals had a midline laparotomy and exposure of the superior mesenteric artery (SMA). Groups A, B and C (sham operation) had no further surgical intervention. The abdominal contents were returned to the abdominal cavity, moistened with warmed normal saline, the abdominal wall approximated and the abdomen covered with a damp swab for the remainder of the experiment (92, 120 and 150 minutes respectively). All other groups underwent occlusion of the SMA by a small atraumatic sprung clip. Cessation of pulsation of the arteries in the small bowel mesentery was confirmed by direct observation. The abdominal contents were returned to the abdominal cavity, moistened with warmed normal saline, the abdominal wall approximated and the abdomen covered with a damp swab. Intestinal ischaemia was maintained for 90 minutes. The SMA was de-occluded by removal of the clip and reperfusion conformed by direct observation of the return of pulsatile flow in the arteries of the small bowel mesentery. Reperfusion was permitted for 2 minutes in Groups D and H, 10 minutes in Groups E and I, 30 minutes in Groups F and J, and 60 minutes in Groups G and K.

At the end of the experiment 2ml blood samples were taken from the superior mesenteric vein (SMV) and aorta into 2ml Plastipak® sterile syringes using 21G Microlance®3 sterile needles (needles and syringes from Becton Dickinson UK Ltd, Cowley, Oxford, UK). Needle and syringe were flushed with heparinised normal saline (25 units lithium heparin salt (Sigma Chemical Co, St Louis, MO, USA) per ml) immediately prior to use.

Blood samples were ejected gently into a 2.0ml capped plastic vial (Safe-Lock, Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany), stored on ice and

Chapter 5: Whole body hypothermia attenuates cytokine and endotoxin responses to intestinal ischaemia-reperfusion

centrifuged at 13000rpm for 5 minutes, within 2 minutes of withdrawal. Plasma was separated immediately, and removed into sterile, endotoxin-free, capped plastic vials (Safe-Lock, Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany). Plasma was centrifuged again at 13000rpm for 5 minutes to separate any inadvertently transferred blood cells then frozen.

Measurement of cytokines and endotoxin

Samples were stored at -80°C until they were processed (median storage time 39 days for cytokine measurement [range 16 – 66 days]; 86 days for endotoxin measurement [range 64 - 112 days]).

Plasma levels of IL 1 β , IL 6, IL 10 and TNF- α were measured using enzyme-linked immunosorbent assay (ELISA; BioSource International, Inc., Camarillo, CA, USA). The manufacturer states that each cytokine kit does not cross-react with other rat cytokines. Briefly, samples were defrosted and transferred to fresh, sterile, pyrogen-free, capped plastic tubes using sterile, pyrogen-free needles and syringes (suppliers as detailed above). Plasma IL 1 β , IL 6, IL 10 and TNF- α concentration were measured by comparison to standard curves of known concentrations of the relevant rat cytokine using an ELISA kit specific to each rat cytokine. Plasma endotoxin concentrations were measured by comparison to a standard curve of known concentration of endotoxin using an endotoxin specific kit (Quantitative Chromogenic Limulus Amebocyte Lysate QCL-1000 Test Kit, BioWhittaker, Inc., Walkersville, MD, USA). All tests were performed in duplicate and a mean concentration used for data analysis.

Statistical analysis

All data are presented as mean \pm SEM. Data were transformed into log₁₀ values to correct for unequal variance between groups and within groups at different time-points. Statistical analysis was performed after testing transformed data for normality by analysis of variance (with Bonferroni post-hoc test for

specified pairs of groups) of \log_{10} transformed data to compare concentrations at 92 and 150 minutes between treatment groups. Linear regression of \log_{10} transformed data was used to examine rate of change in concentration, and to compare rate of change in concentration between treatment groups and sample sites. Two-way analysis of variance was used to examine time and treatment effects in the absence of straightforward linear changes over experimental time. Statistical significance was set at $P < 0.05$. All statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, CA, USA).

Results

Figure 5.1 shows plasma IL-6 concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR.

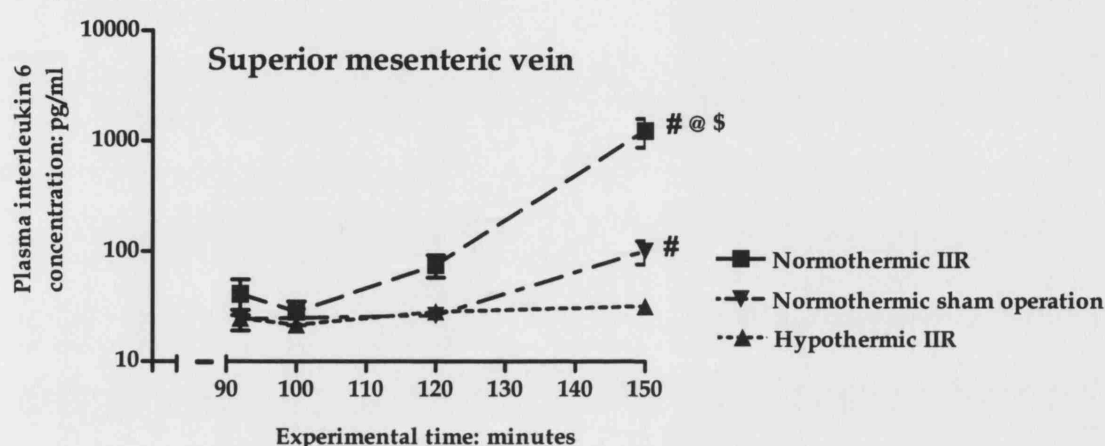


Figure 5.1: Plasma IL-6 concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

@ $P < 0.05$ versus normothermic sham operation at 150 minutes

\$ $P < 0.05$ versus hypothermic intestinal ischaemia-reperfusion at 150 minutes

$P < 0.05$ (increase in log₁₀ IL-6 over experimental time)

$P < 0.05$ (rate of increase over time equal between groups)

Overall, IL-6 concentration in the SMV increased after normothermic IIR and normothermic sham operation but not after hypothermic IIR. SMV IL-6 concentration was not significantly different between treatment groups at 92 minutes. At 150 minutes IL-6 concentration was higher after normothermic IIR than after both normothermic sham operation and hypothermic IIR. IL-6 concentration after hypothermic IIR was not significantly different to that after normothermic sham operation at 150 minutes. A significant rise in SMV IL-6

concentration with increasing reperfusion time was seen after normothermic IIR but not after hypothermic IIR. A less marked but still significant rise in IL-6 concentration with increasing experimental time was seen after normothermic sham operation. The rate of increase in IL-6 observed in this experiment was **not** equal between treatment groups – the rate of rise seen after normothermic IIR was more rapid than that seen after normothermic sham operation.

Figure 5.2 shows plasma IL-6 concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR.

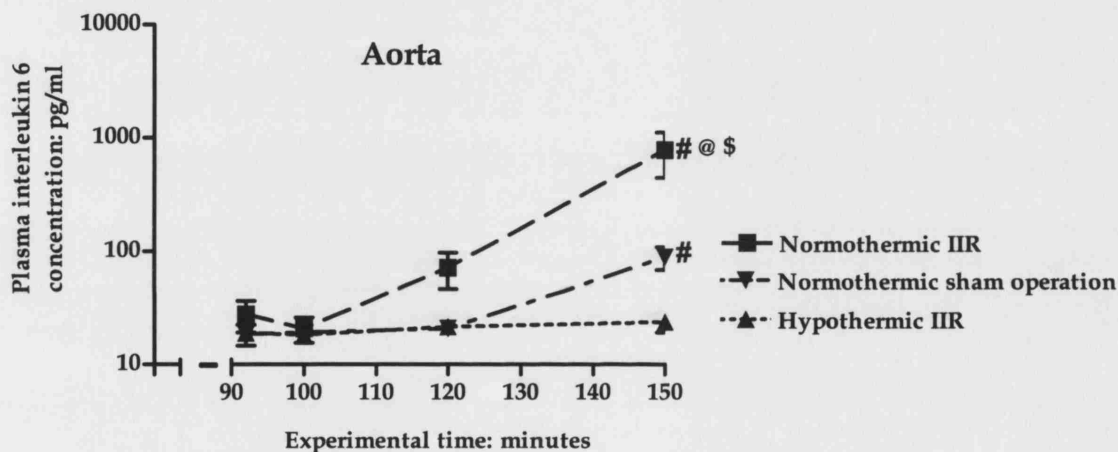


Figure 5.2: Plasma IL-6 concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

@ $P < 0.05$ versus normothermic sham operation at 150 minutes

\$ $P < 0.05$ versus hypothermic intestinal ischaemia-reperfusion at 150 minutes

$P < 0.05$ (increase in log₁₀ IL-6 over experimental time)

$P < 0.05$ (rate of increase over time equal between experimental groups)

Overall, the pattern of change in IL-6 concentration seen in the aorta is very similar to that seen in the SMV – IL-6 concentration increased after normothermic IIR and normothermic sham operation but not after hypothermic IIR. Aortic IL-6

concentration was not significantly different between treatment groups at 92 minutes. At 150 minutes IL-6 concentration was higher after normothermic IIR than after both normothermic sham operation and hypothermic IIR. IL-6 concentration after hypothermic IIR was less than that after normothermic sham operation at 150 minutes. A significant rise in aortic IL-6 concentration with increasing reperfusion time was seen after normothermic IIR but not after hypothermic IIR. A less marked but still significant rise in IL-6 concentration with increasing experimental time was seen after normothermic sham operation. The rate of increase in IL-6 observed in this experiment was **not** equal between treatment groups – that seen after normothermic IIR was more rapid than that seen after normothermic sham operation.

Within each treatment group there was no significant difference in rate of change in IL-6 concentration with increasing experimental time between superior mesenteric venous and aortic samples.

Porto-systemic difference (SMV minus aortic) in concentration of IL-6 was not significantly different at 92 and 150 minutes of experimental time, and did not vary significantly with increasing experimental time for all treatment groups.

Figure 5.3 shows plasma IL-10 concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR.

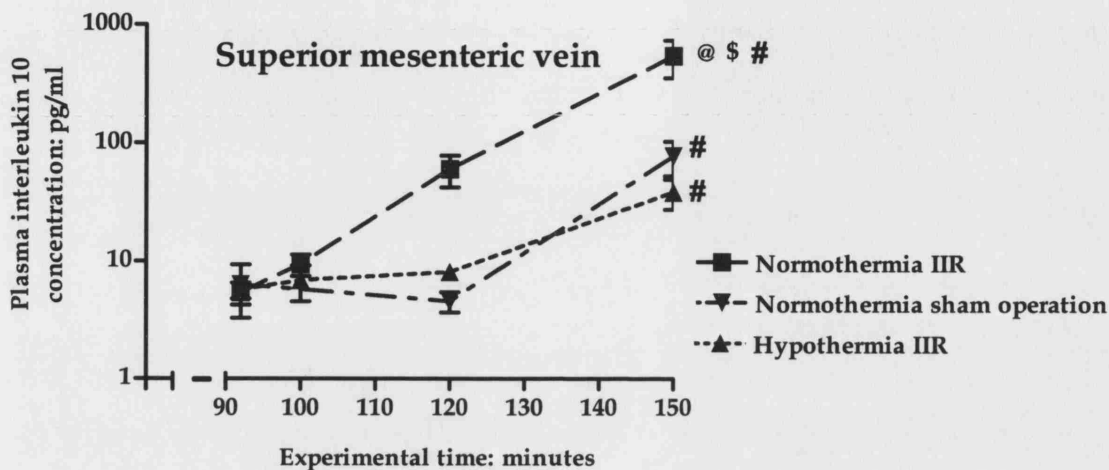


Figure 5.3: Plasma IL-10 concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

@ $P < 0.05$ versus normothermic sham operation at 150 minutes

\$ $P < 0.05$ versus hypothermic intestinal ischaemia-reperfusion at 150 minutes

$P < 0.05$ (increase in log₁₀ IL-10 over experimental time)

$P < 0.05$ (rate of increase over time equal between groups)

Overall, SMV IL-10 concentration increased with increasing experimental time in all treatment groups but concentrations after normothermic sham operation and hypothermic IIR were consistently lower than those seen after normothermic IIR. SMV IL-10 concentration was not significantly different between treatment groups at 92 minutes. At 150 minutes IL-10 concentration was higher after normothermic IIR than after both normothermic sham operation and hypothermic IIR. IL-10 concentration after hypothermic IIR was not significantly different to that after normothermic sham operation at 150 minutes. A significant

rise in SMV IL-10 concentration with increasing reperfusion time was seen in all treatment groups. The rate of increase in IL-10 observed in this experiment was **not** equal between treatment groups – that seen after normothermic IIR was more rapid than that seen after normothermic sham operation and hypothermic IIR.

Figure 5.4 shows plasma IL-10 concentrations in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR.

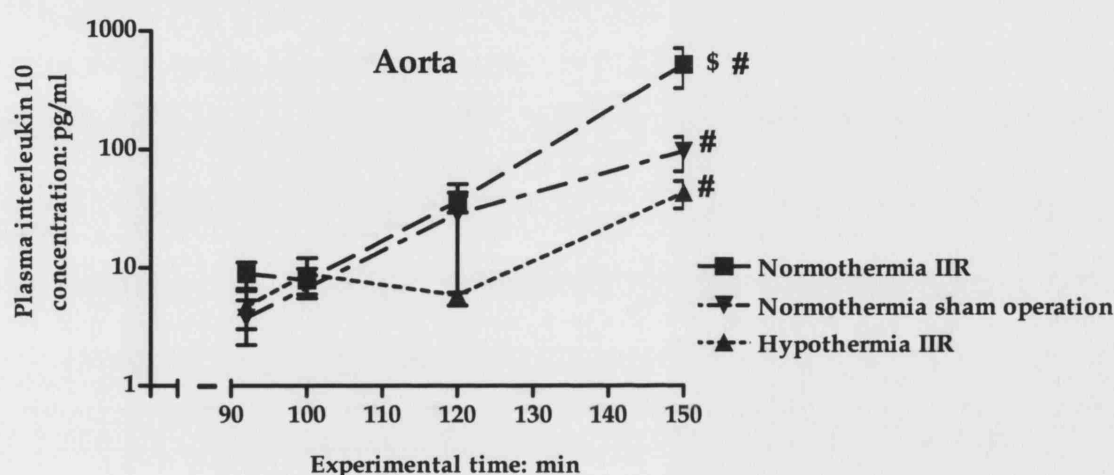


Figure 5.4: Plasma IL-10 concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

\$ $P < 0.05$ versus hypothermic intestinal ischaemia-reperfusion at 150 minutes

$P < 0.05$ (increase in log₁₀ IL-10 over experimental time)

The pattern of change in IL-10 concentration seen in the aorta is very similar to that seen in the SMV with an increase in IL-10 concentration with increasing experimental time in all treatment groups. Again normothermic sham operation and hypothermic IIR concentrations were lower than those seen after normothermic IIR. Aortic IL-10 concentration was not significantly different between treatment groups at 92 minutes. At 150 minutes IL-10 concentration was higher after normothermic IIR than after hypothermic IIR. IL-10 concentration

after hypothermic IIR was not significantly different to that after normothermic sham operation at 150 minutes. A significant rise in aortic IL-10 concentration with increasing reperfusion time was seen in all treatment groups. The rate of increase in aortic IL-10 observed in this experiment was equal between treatment groups.

Within each treatment group there was no significant difference in rate of change in IL-10 concentration with increasing experimental time between superior mesenteric venous and aortic samples.

Porto-systemic difference in concentration of IL-10 (superior mesenteric venous minus aortic) was not significantly different at 92 and 150 minutes of experimental time, and did not vary significantly with increasing experimental time for all treatment groups.

Figure 5.5 shows plasma TNF- α concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR.

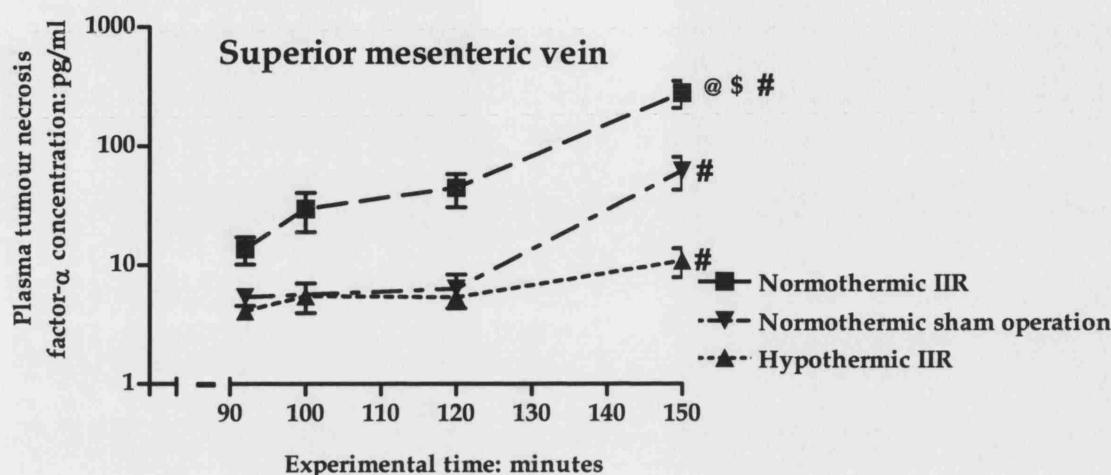


Figure 5.5: Plasma TNF- α concentration in the SMV after normothermic IIR, normothermic sham or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

@ $P < 0.05$ versus normothermic sham operation at 150 minutes

\$ $P < 0.05$ versus hypothermic intestinal ischaemia-reperfusion at 150 minutes

$P < 0.05$ (increase in log₁₀ tumour necrosis factor- α over experimental time)

$P < 0.05$ (rate of increase over time equal between groups)

Overall, TNF- α concentration in the SMV increased with increasing experimental time in all treatment groups, but levels seen after normothermic sham operation and hypothermic IIR were consistently lower than those seen after normothermic IIR. SMV TNF- α concentration was not significantly different between treatment groups at 92 minutes. At 150 minutes TNF- α concentration was higher after normothermic IIR than after both normothermic sham operation and hypothermic IIR. TNF- α concentration after hypothermic IIR was lower than that after normothermic sham operation at 150 minutes. A significant rise in SMV

TNF- α concentration with increasing reperfusion time was seen in all treatment groups. The rate of increase of TNF- α concentration observed in this experiment was **not** equal between treatment groups – that seen after normothermic IIR was more rapid than that seen after hypothermic IIR.

Figure 5.6 shows plasma TNF- α concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR.

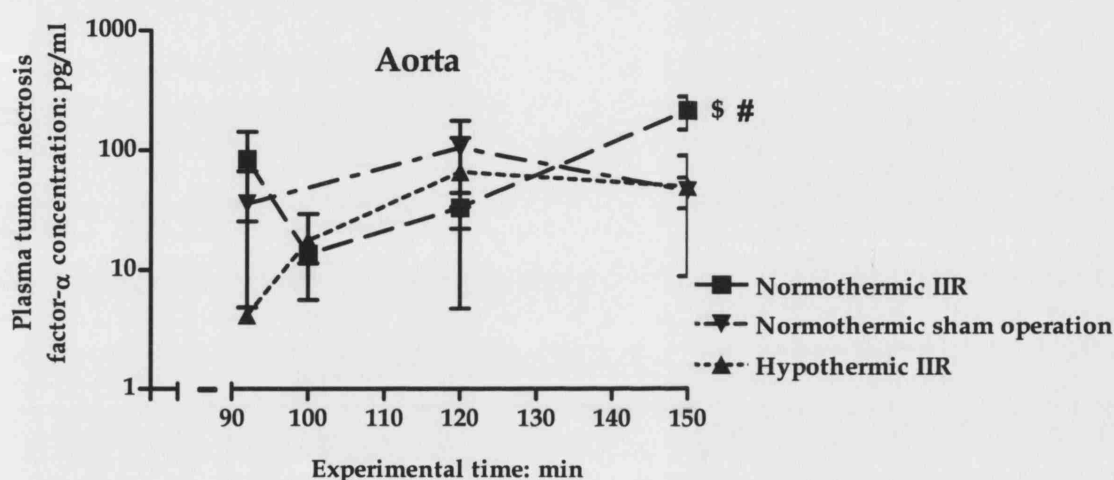


Figure 5.6: Plasma TNF- α concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log10 scale on y-axis

N = 6 – 12 per group per time point

\$ P < 0.05 versus hypothermic intestinal ischaemia-reperfusion at 150 minutes

P < 0.05 (increase in log10 tumour necrosis factor- α over experimental time)

The pattern of change in TNF- α concentration seen in the aorta is **not** the same as that seen in the SMV. Normothermic sham operation and hypothermic IIR groups both show an initial rise in TNF- α which appears possibly to be in decline by 150 minutes. In contrast, normothermic IIR shows an initial drop followed by a sustained rise which does not appear to have peaked at 150 minutes. Aortic TNF- α concentration was not significantly different between treatment

groups at 92 minutes. At 150 minutes TNF- α concentration was higher after normothermic IIR than after hypothermic IIR. A significant rise in aortic TNF- α concentration with increasing reperfusion time was seen after normothermic IIR only.

Within each treatment group there was no significant difference in rate of change in TNF- α concentration with increasing experimental time between SMV and aortic samples.

Figure 5.7 shows the porto-systemic difference (SMV minus aortic) in concentration of TNF- α over experimental time after normothermic IIR, normothermic sham operation or hypothermic IIR. Positive values indicate an excess of TNF- α in the SMV compared to the aorta.

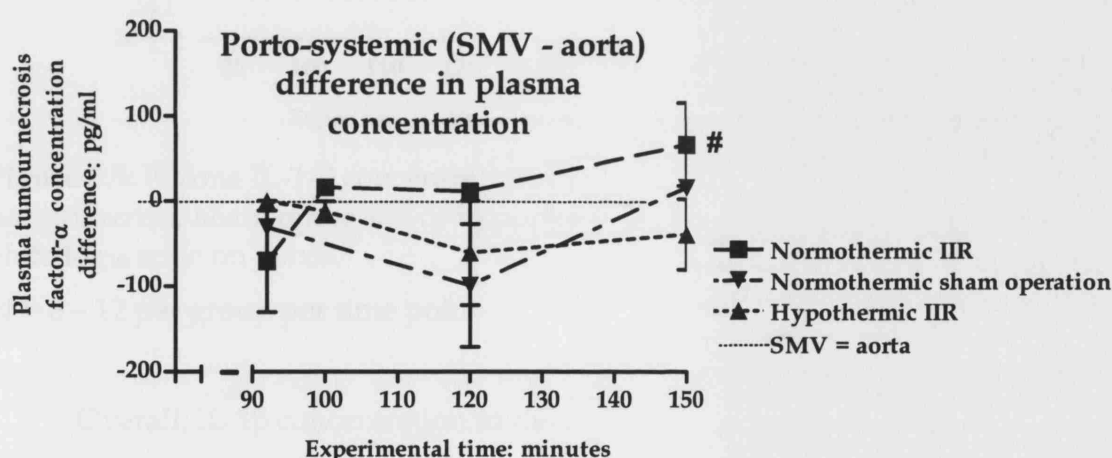


Figure 5.7: Plasma TNF- α concentration difference between the SMV and the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR

N = 6 – 12 per group per time point

$P < 0.05$ (increase in TNF- α porto-systemic difference over experimental time)

Overall normothermic IIR results in a steady increase in porto-systemic difference in TNF- α , but normothermic sham operation and hypothermic IIR do not result in a consistent porto-systemic difference in TNF- α . The porto-systemic difference in TNF- α was not significantly different between treatment groups at 92

and 150 minutes of experimental time. A rise in porto-systemic difference in TNF- α concentration with increasing experimental time was seen after normothermic IIR only.

Figure 5.8 shows plasma IL-1 β concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR.

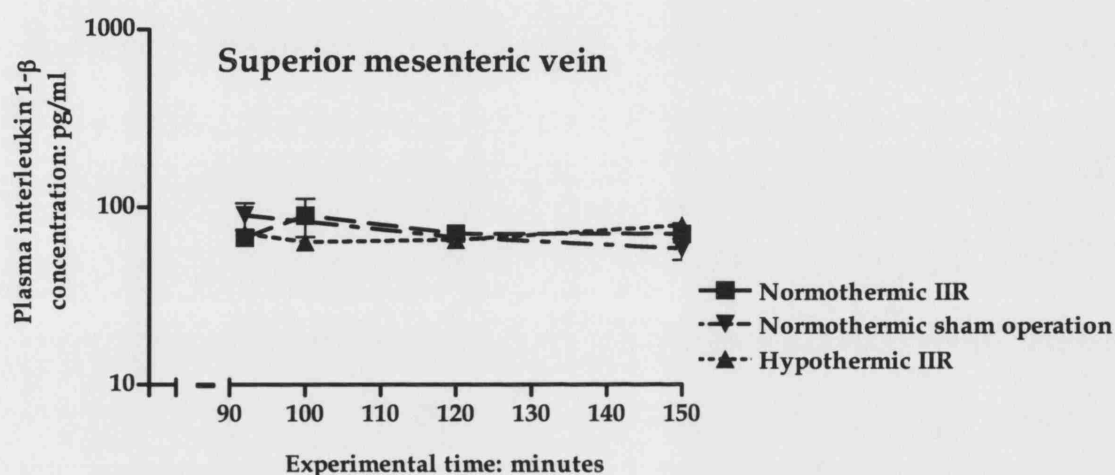


Figure 5.8: Plasma IL-1- β concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

Overall, IL-1 β concentration in the SMV showed no differences between treatment groups and did not change with experimental time.

Figure 5.9 shows plasma IL-1 β concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR.

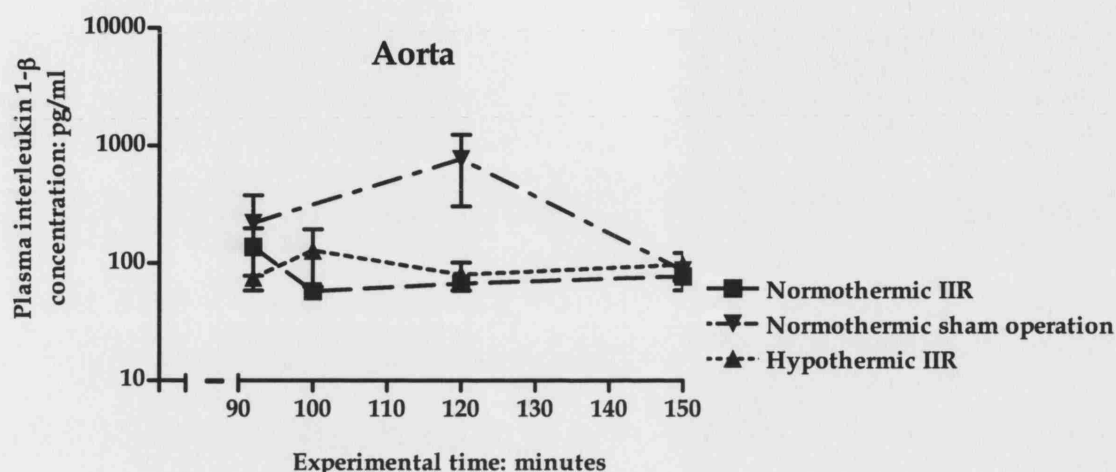


Figure 5.9: Plasma IL-1- β concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

Overall IL-1 β concentration in the aorta did not show consistent changes with experimental time in any treatment group.

Within each treatment group there was no significant difference in rate of change in IL-1 β concentration with increasing experimental time between SMV and aortic samples.

Porto-systemic difference in concentration of IL-1 β (superior mesenteric venous minus aortic) was not significantly different at 92 and 150 minutes, and did not vary significantly with increasing experimental time for all treatment groups.

Figure 5.10 shows plasma endotoxin concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR.

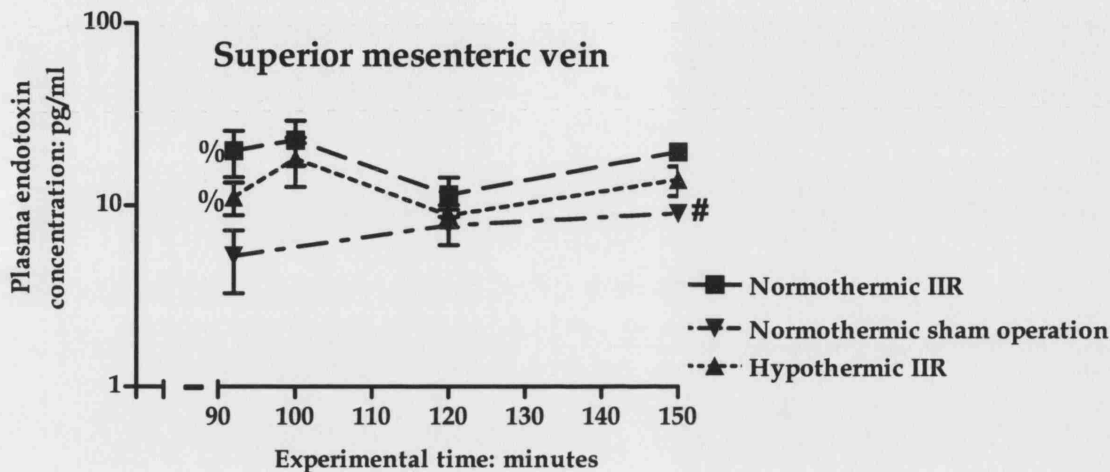


Figure 5.10: Plasma endotoxin concentration in the SMV after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

% $P < 0.05$ versus normothermic sham operation at 92 min

$P < 0.05$ (increase in log₁₀ endotoxin over experimental time)

$P < 0.05$ (sham operation \neq intestinal ischaemia-reperfusion)

Overall, an increase in endotoxin is seen in the SMV after normothermic IIR and hypothermic IIR after 2 minutes of reperfusion compared to sham operation, but these very early differences are not sustained at 150 minutes of experimental time/60 minutes of reperfusion. Sham operation results in a steady increase in SMV endotoxin with experimental time, but IIR results in a more complicated pattern of endotoxin variation over time with high initial levels showing a further rise at 10 minutes, then falling at 30 minutes and rising again at 60 minutes of reperfusion.

Endotoxin concentration in the SMV at 92 minutes of experimental time was significantly higher after both normothermic and hypothermic IIR than after sham operation. No significant differences were apparent after 150 minutes of experimental time. Only sham operation showed a consistent rise in endotoxin with increasing experimental time.

Two-way analysis of variance indicated no significant difference in SMV endotoxin concentration between normothermia and hypothermia after IIR, but a significant difference was found between IIR and sham operation ($P = 0.0002$).

Figure 5.11 shows plasma endotoxin concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR.

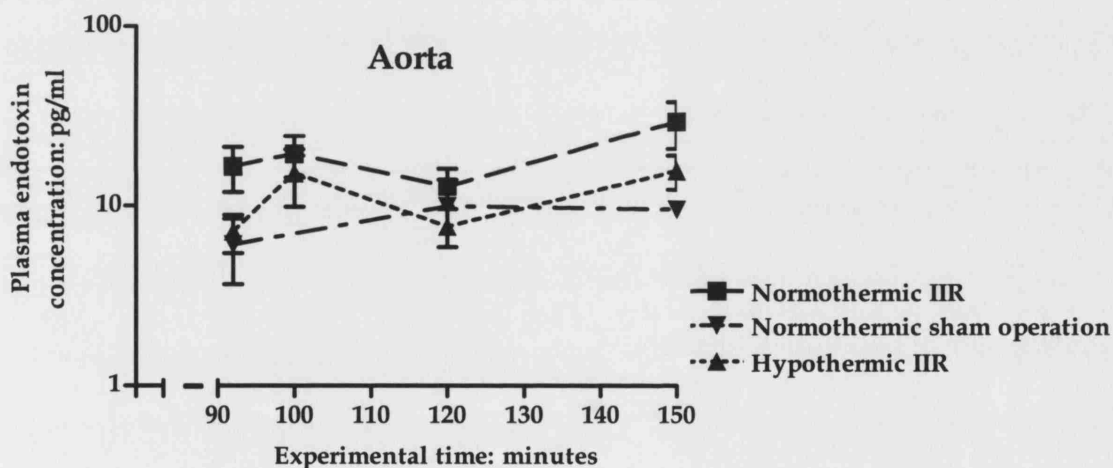


Figure 5.11: Plasma endotoxin concentration in the aorta after normothermic IIR, normothermic sham operation or hypothermic IIR

Note log₁₀ scale on y-axis

N = 6 – 12 per group per time point

$P < 0.05$ (change in log₁₀ endotoxin over experimental time with IIR at normothermia and hypothermia)

$P < 0.05$ (hypothermic IIR < normothermic IIR)

$P < 0.05$ (sham operation \neq IIR)

Overall, sham operation results in a low level of aortic endotoxin with no variation with experimental time. IIR results in a complicated pattern of endotoxin variation over time in the aorta with initial levels indicating a further rise at 10 minutes, a fall at 30 minutes and a second rise at 60 minutes of reperfusion.

Endotoxin concentration in the aorta at 92 and 150 minutes of experimental time was not significantly different between treatment groups. No consistent changes with experimental time were seen in any treatment group.

Two-way analysis of variance indicated a significant difference in aortic endotoxin concentration between normothermia and hypothermia in IIR treatment groups, with hypothermia resulting in consistently lower levels of aortic endotoxin at all sampling times ($P = 0.0190$). A significant difference was also found between IIR and sham operation ($P = 0.0028$).

Within each treatment group there was no significant difference in rate of change in endotoxin concentration with increasing experimental time between SMV and aortic samples.

Figure 5.12 shows the porto-systemic difference (SMV minus aortic) in concentration of endotoxin over experimental time after normothermic IIR, normothermic sham operation or hypothermic IIR. Positive values indicate an excess of endotoxin in the SMV compared to the aorta.

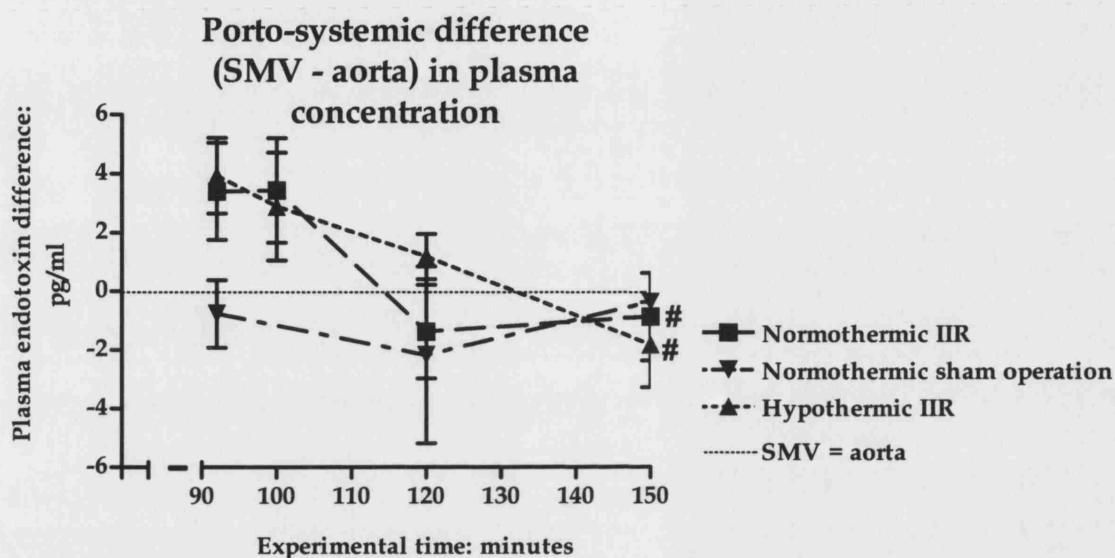


Figure 5.12: Plasma endotoxin porto-systemic difference in concentration after normothermic IIR, normothermic sham operation or hypothermic IIR

N = 6 - 12 per group per time point

$P < 0.05$ (decrease in endotoxin difference over experimental time)

Overall, sham operation results in a porto-systemic difference in endotoxin which does not differ significantly from zero (i.e. SMV and aortic equivalence) at any time point measured, and shows no consistent change with increasing experimental time. Both normothermic IIR and hypothermic IIR result in a fall porto-systemic difference in endotoxin with increasing experimental time.

No significant differences between treatment groups were seen at 92 or 150 minutes of experimental time. A fall in porto-systemic difference in endotoxin over experimental time was seen with IIR in both normothermic and hypothermic

groups. No consistent change with experimental time was seen with sham operation.

Discussion

This experiment measured cytokine and endotoxin levels in the portal and systemic circulations after normothermic IIR, normothermic sham operation and whole body hypothermic IIR in an adult rat model. Overall, whole body hypothermia suppressed the release of cytokines into the portal and systemic circulations, and reduced the endotoxin load reaching the systemic circulation. It was interesting to demonstrate that whole body hypothermia reduced circulating levels of cytokines at 150 minutes of experimental time to levels at or below those seen with normothermic sham operation. Because of this, the decision to omit a hypothermic sham group was unfortunate in that a direct comparison of the effect of hypothermia under sham conditions might have clarified the effect of sham operation in the normothermic group. It was also interesting to note that the changes in cytokine concentrations observed occurred with the same time-course in both the portal and systemic circulations – i.e. there was no porto-systemic delay in the inflammatory response.

IL-6, IL-10 and TNF- α all showed a broadly similar pattern of change after normothermic IIR, with rises in the SMV and aorta with increasing reperfusion time. This echoes findings in the published literature (Bathe et al, 1998; Grotz et al, 1999). The magnitude of the rise in cytokine levels over time indicates an exponential increase in circulating cytokine levels in both the portal and systemic circulations. In the portal circulation this would be expected in a positive feedback situation in which enterocytes and tissue-associated leukocytes activated by the original insult release cytokines locally. These locally concentrated cytokines attract circulating leukocytes passing through the tissue, triggering their activation and subsequent migration into the local tissue. Enhanced leukocyte adhesion and

migration has been shown to occur in venules after exposure to TNF- α (Zeng, 2002). Once *in situ* within intestinal interstitial tissue, recruited leukocytes in turn release cytokines locally resulting in a positive feedback loop. If circulating leukocytes within the intestine are activated by locally released cytokines but do not migrate immediately into the local tissues after activation, the inflammatory response is relayed on to the liver. Cytokines and other bioactive molecules produced in the intestine are also delivered to the liver in the portal circulation and have been shown to be able to activate Kupffer cells (Towfigh et al, 2000), giving rise to intra-hepatic leukocyte activation and cytokine production. This relaying of activation from the gut to the liver may be hypothesised to result in both a delay in the appearance of increased levels of cytokines in the systemic circulation as the signal is passed on within the liver, and an amplification of the inflammatory signal arising in the gut (Ogle et al, 1994). If the liver becomes a cytokine-generator, the inflammatory response will affect the whole animal as cytokines and other inflammatory mediators are released into the systemic circulation.

As no difference was found in the time-course of the rise of cytokine concentration between the samples taken from the superior mesenteric vein and the aorta for any of the cytokines measured, the hypothesised delay in the appearance of cytokines in the systemic circulation has not been supported by this experiment. A reason for this lack of evidence of a clear porto-systemic delay may include the level of cytokine concentration required within the liver to trigger Kupffer cell activation and hepatic cytokine release. If the triggering cytokine concentration required is very low, the Kupffer cell population may reach sufficient levels of activation and become cytokine-generating very rapidly, thus masking the hypothesised intra-hepatic delay. If this is the case, it may be expected that ongoing delivery of gut-activated leukocytes and positive feedback within the liver would result in an overshoot of systemic cytokine levels as the liver amplifies the cytokine signal from the intestines. This was not observed

either – levels measured in the systemic and portal circulations remained closely related throughout the experiment. That this was not seen may be partially attributed to the changes in hepatic inflow measured during the reperfusion of previously ischaemic gut in Chapter 4 of this thesis (pages 173 – 220). If the contribution of hepatic inflow arising from the gut decreases, then the inflow of gut-activated leukocytes will also decrease, reducing the delivery of external stimulus to Kupffer cells in the liver. Also if total hepatic inflow falls, as was noted during the later stages of IIR, then hepatic outflow will also fall, reducing delivery of liver-derived cytokines to the systemic circulation and thus masking the increased rate of production of cytokines within the liver by reducing the rate of delivery to the systemic circulation.

Surprisingly, sham operation resulted in a similar pattern of cytokine changes to those seen with IIR, but to a lesser degree. This is at odds with results in the published literature (Bathe et al, 1998). The results from this experiment imply that prolonged normothermic general anaesthetic with sham operation only may be considered to generate a mild inflammatory response by itself. This should therefore be taken into account in the design of future experiments.

The lack of consistent change in IL 1- β levels with any of the treatments given was unexpected. Other groups have shown a change in IL 1- β concentration with similar IIR based insults (Bitterman et al, 1991; Fu et al, 1999; Ledebøer, 2002; Wyble et al, 1996). The explanation for this might relate to several different factors. For example, the timing of the samples (IL 1- β expression is thought to rise later in the inflammatory response); the type of test-kit used (ELISA kits measure unbound IL 1- β but plasma contains both specific and non-specific IL 1- β inhibitors which can bind IL 1- β); or the relative level of IL-10 (increases in IL 1- β induced by lipopolysaccharide stimulation can be suppressed by IL-10). Further experiments using this model with delayed sampling, a measuring technique independent of

plasma binding of IL 1- β and/or suppression of IL-10 release may show a more informative pattern of changes of IL 1- β levels.

Endotoxin was measurable in the portal and systemic circulations with normothermic sham operation, but levels were consistently higher after IIR than after sham operation in both the portal and systemic circulations. This supports the hypothesis that plasma endotoxin levels rise after IIR both locally and systemically more than can be attributed to sham operation alone (Gathiram et al, 1989). Exposure of Kupffer cells to lipopolysaccharide (LPS) has been shown to result in a dose-dependent increase in TNF- α production via a CD14-mediated pathway (Su, 2002). In this experiment systemic levels of TNF- α did not correlate with portal endotoxin levels. This is more likely to reflect the complexity of the whole animal model used here than provide conflicting evidence for Su's cell culture-based study.

Whole body hypothermia profoundly changed the pattern of changes in cytokine levels seen in both the SMV and aorta after IIR. More subtle, but still significant, differences were seen in endotoxin levels after moderately hypothermic IIR. Whole body hypothermia appears to have a differential effect on pro- and anti-inflammatory cytokines. The rise in pro-inflammatory cytokines IL-6 and TNF- α with increasing reperfusion time was abolished in the SMV and aorta, and at 60 minutes of reperfusion, some levels were even lower than with normothermic sham operation (in the SMV for TNF- α , and in the aorta for IL 6). A rise with increasing reperfusion time was seen with anti-inflammatory cytokine IL 10, but levels reached at 60 minutes of reperfusion were only equivalent to normothermic sham. A potential mechanism for this differential action of moderate hypothermia is suggested by the work of Adanin et al (Adanin, 2002) who have shown that inhibition of adenosine deaminase tempers but does not obliterate pro-inflammatory responses triggered by exposure to LPS.

It is interesting to note that whole body hypothermia significantly reduced endotoxin levels in the aorta during intestinal reperfusion, but not in the SMV. This suggests that whole body hypothermia does not prevent the delivery of endotoxin from the gut into the portal circulation, but does enhance the removal of endotoxin before it reaches the systemic circulation. Therefore this experiment shows that hypothermia does not result in total immune paralysis, as is occasionally feared (Akriotis et al, 1985).

Although endotoxin levels showed a more complex pattern than the expected progressive rise with increasing reperfusion time in either the SMV or aorta, the porto-systemic difference over the duration of the experiment did decrease with increasing reperfusion time after both normothermic and hypothermic IIR. This demonstrates that although endotoxin levels were higher in the SMV than in the aorta soon after the onset of reperfusion, with increasing reperfusion time successful removal of the excess endotoxin load was progressively compromised. This affected both normothermic and hypothermic IIR animals. It is interesting to note that hypothermic IIR apparently prolonged the period of intestinal reperfusion for which aortic endotoxin levels were less than SMV levels (hypothermic IIR difference is still positive at 30 minutes of intestinal reperfusion whilst normothermic IIR porto-systemic difference is zero or negative). By the end of the experiment portal and systemic levels were not significantly different at both temperatures. This suggests that the ability of whole body hypothermia to preserve the effective elimination of endotoxin is reperfusion time-dependent and exhaustible, which will have implications for its potential use as a therapeutic strategy. Work by Drewe (Drewe, 2001) suggests both paracellular and transcellular transport of endotoxin within the liver. Clarification of the mechanism by which endotoxin is removed from the portal circulation within the liver may give an insight into the beneficial but exhaustible action of whole body hypothermia in supporting hepatic endotoxin clearance.

The major impact of whole body hypothermia on cytokine and endotoxin levels after IIR may be explained by several different possible mechanisms, which could be acting concurrently.

A direct effect on leukocytes of whole body hypothermia could be hypothesised. Hypothermia may act to stabilise leukocytes (Chatzipanteli et al, 2000; Toyoda et al, 1996), making tissue leukocytes less likely to become activated, and circulating leukocytes less likely to marginate (Ishikawa et al, 1999; Rowin et al, 2001) and migrate into damaged tissue (Biggar et al, 1984). Within the intestine this will result in attenuated release of cytokines locally compared to an identical insult under normothermic conditions as the positive feedback loop described above is limited by the impaired response of individual leukocytes. As hypothermia was applied to the whole animal in these experiments, these effects would also pertain to the liver, thus reducing the relay of the inflammatory insult from the gut to the systemic circulation. Systemic proliferation of the inflammatory response would also be limited. This could explain the reduction in cytokine levels in both the portal and systemic samples, but not the maintenance of endotoxin clearance by hypothermic animals. A differential leukocyte response to ischaemia and endotoxin when hypothermic could be included in the hypothesis to explain why the less active leukocytes are still able to clear LPS.

With a global reduction in leukocyte activation and therefore in overall pro-inflammatory conditions, one might expect less capillary leak and therefore less local tissue oedema. The mechanism causing increased micro-vascular permeability has been shown to be independent of leukocyte adhesion and migration in a mesenteric venule exposed to TNF- α (Zeng, 2002). However, increased local micro-vascular permeability resulting in marked tissue oedema has been shown after IIR in whole animal models similar to this one (Souza et al, 2000; Ward et al, 2000) which would provide an obstruction to blood flow within the intestine. If a reduction in local pro-inflammatory conditions decreased capillary

leak and reduced local tissue oedema, the maintenance of flow through the intestine after IIR could result in a further reduction in opportunity for the activation and migration of leukocytes in the intestine as overall velocity of flow would be higher. This would reduce the positive feedback of local release of cytokines by activated gut-associated leukocytes as outlined above.

A different hypothesis would suggest an effect of whole body hypothermia on cytokine signalling between cells. In this case, leukocytes may become activated and migrate as if under normothermic conditions, but are unable to interpret inter-cellular signals appropriately and therefore under-produce cytokines. This may help to explain the apparent differential effect of hypothermia on pro-and anti-inflammatory cytokines in this experiment, and the relative preservation of endotoxin elimination. As suggested above, this may involve adenosine (Adanin et al, 2002).

These suggested mechanisms would interplay and it is not possible to characterise the actual site of action of whole body hypothermia from this experiment. Leukocytes may not be the only cell-type to become less likely to produce cytokines when hypothermic – enterocytes and hepatocytes may be similarly affected thus reducing the portal and systemic cytokine load further.

Alternatively, if whole body hypothermia reduced the local damage caused by IIR within the intestine, the overall insult will be reduced and therefore the cytokine response and endotoxin load may be expected to be blunted.

Mechanisms by which this may be accomplished include lowering the metabolic rate of intestinal cells or otherwise increasing the resilience of intestinal cells to intestinal ischaemia-reperfusion injury. The impact of low oxygen tension, poor glucose delivery and waste product stagnation would therefore be ameliorated. Unfortunately, evidence is available that intestinal damage after IIR is in no way ameliorated by whole body hypothermia (Vejchapipat et al, 2000). In the same vein, it is difficult to argue that whole body hypothermia would reduce the local

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effects of IIR to an insult less than sham operation at normothermia, which has been shown in this experiment to have a significant impact on portal and systemic cytokine and endotoxin levels. This experiment did not attempt to address the impact of whole body hypothermia on sham operation only, and this may be an interesting area of future study.

Summary and Conclusions

An existing animal model was used to document changes in portal and systemic cytokine and endotoxin concentrations during normothermic sham operation of up to 150 minutes or 90 minutes of intestinal ischaemia followed by up to 60 minutes of intestinal reperfusion under either normothermic or hypothermic conditions. Normothermic IIR resulted in raised portal and systemic cytokine and endotoxin levels. The endotoxin porto-systemic difference fell with increasing reperfusion time.

Whole body hypothermia profoundly reduced cytokine levels in both portal and systemic circulations after IIR, in some cases to less than those seen with normothermic sham operation. Endotoxin levels were reduced in the systemic circulation only, and porto-systemic equivalence was delayed. The mechanism of this protective influence of whole body hypothermia might involve the stabilisation of tissue and circulating leukocytes and/or a reduction in the severity of the intestinal insult.

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Chapter 6: Near Infrared Spectroscopy of the intestine during intestinal ischaemia-reperfusion

Introduction

Intestinal ischaemia can be very difficult to diagnose in a clinical setting (Bjorck et al, 2002), especially in the presence of other major pathology causing physiological compromise – for example the critically sick patient ventilated on the intensive care unit with severe sepsis - due to a paucity of specific symptoms and signs in this condition. The importance of diagnosis is exemplified by the critically sick intensive care unit patient in whom the consequences of either a missed diagnosis or an unnecessary operation are equally serious. Diagnosis in these circumstances usually relies on non-specific evidence of poor tissue perfusion [rising lactate levels (Duke et al, 1997), increased lactate-to-pyruvate ratio (Hotchkiss and Karl, 1992), increasing metabolic acidosis (Jamieson et al, 1982)] or tissue injury [high inorganic phosphate levels in serum (Sodhi and Arora, 1993) or peritoneal fluid (Sawer et al, 1978), raised intestinal fatty acid binding protein (Gollin et al, 1993)]. Monitoring developed to address this issue to date includes gastric (Reilly and Bulkley, 1993), jejunal (Schlichting and Lyberg, 1995) and rectal (Koivusalo et al, 2000) tonometry, Doppler blood velocity recordings from intestinal blood vessels (Okada et al, 2001), and repeated abdominal X-radiography or ultrasound [low sensitivity unless intra-mural or intraportal gas is present (Santulli et al, 1975; Kliegman and Fanaroff, 1984)]. Mesenteric angiography (Bjorck et al, 2002) and contrast enhanced computerized tomography or magnetic resonance imaging may also be of use (Mottet et al, 1996; Bjorck et al, 2002). Each of these methods represents a mixture of risk and benefit to the patient. Tonometry can give some information about regional respiration by estimating

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local bicarbonate production, but information yield is intermittent, relatively slow (up to 40 minutes of equilibration per reading) and requires the patient to tolerate an intestinal probe at all times. Doppler blood velocity recordings have the advantage of being non-invasive, but do not give information about blood flow into an organ unless a series of assumptions about vessel diameter and the laminar nature of flow are made, which may or may not be reasonable. Recordings can be difficult to obtain, are intermittent, and are operator-dependent. The critical velocity or calculated flow rate may be difficult to ascertain for individual patients, giving rise to problems with the interpretation of data and its use in clinical decision making. Repeated abdominal X-radiography or ultrasound will also provide intermittent information and can only identify late changes in intestinal ischaemic injury (pneumatosis intestinalis, gas in the portal vein and liver). X-radiography carries radiation exposure risk and ultrasonography is operator-dependent. Interpretation of either type of images can be difficult.

Purely non-invasive, non-radiation based methods of monitoring patients at risk of intestinal ischaemia have not yet been commercially developed, but near-infrared spectroscopy (NIRS) is a strong candidate. The technique is based on the change in absorbance of haemoglobin in the near-infrared range (700 – 1000nm) depending on oxygenation status. NIRS has a number of advantages over other methods of detecting changes in perfusion. It carries no radiation exposure or other physical risk. It can be used continuously to provide a real-time readout of tissue oxygenation index (ratio of oxygenated haemoglobin to total haemoglobin within interrogated tissue). No items are consumed during use, making running costs very low after initial investment in the equipment. Tissue penetration has been recorded at up to 8cm (Elwell, 1995) and work is ongoing to solve the theoretical problems concerning path-length and light scatter within tissue that have prevented the absolute quantification of tissue oxyhaemoglobin and deoxyhaemoglobin levels to date (Wahr et al, 1996). Other complicating factors

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include movement of organs with respiration and within the abdomen during a NIRS study, which can make changes in NIRS signals difficult to interpret with certainty, and the attenuation of near infrared light by skin (especially coloured skin – melanin absorbs light in the near-infrared spectrum) and subcutaneous tissue (Feng et al, 2001).

NIRS is already established a practical means of monitoring neonatal cerebral haemodynamics, especially in the sick child as it can be performed continuously at the cotside (Edwards et al, 1988), and it may have a role in assisting in prognostication in the perinatally asphyxiated child (Wyatt, 1993). NIRS has been used in many other clinical contexts – liver transplantation (Tokuka et al, 1994), peripheral vascular disease (Kooijman et al, 1997) and breast imaging (Shah et al, 2001) and further experimental work has explored assessment of acute lung injury (Shibata et al, 1999), renal ischaemia-reperfusion injury (Vaughan et al, 1995), burns (Sowa et al, 2001) and pedicled grafts (Stranc et al, 1998). These applications have challenges different from those of NIRS of the gut – organs are relatively fixed or can be anchored; studies were experimental and invasive; and the tissue examined is relatively homogeneous. NIRS of the intestines has already been used in an uncontrolled clinical setting to detect changes in tissue haemoglobin oxygenation status in the infant abdomen (Fortune et al, 2001b; Fortune et al, 2001a; Petros et al, 1999). In experimental work, intra-gastric tissue haemoglobin oxygen saturation measured by NIRS has been shown to correlate well with flow in the superior mesenteric artery (SMA) in a pig haemorrhage model (Cohn et al, 2001) and in a pig model of abdominal hypertension with and without haemorrhage and resuscitation (Varela et al, 2001). It is interesting that this correlation was shown despite recording tissue haemoglobin oxygenation in the stomach which is not supplied by the SMA, and it is not clear how the non-independent nature of the readings from each animal were dealt with statistically. However, it appears that NIRS of the intestinal tract is possible and, for the reasons

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outlined above, it is a worthwhile research goal to establish its practicality in the intestines. It is hypothesized that NIRS of the intestines will yield reproducible signals that reflect real-time changes in haemoglobin oxygenation state within the intestines and/or intestinal blood influx or efflux during intestinal ischaemia-reperfusion (IIR) in an established rat model.

The aim of the first experiment was to establish if NIRS optodes can be directly applied to the intestines to detect changes in oxyhaemoglobin and deoxyhaemoglobin signals from small bowel during experimentally induced small bowel ischaemia-reperfusion in suckling rats.

The aim of the second experiment was to establish whether NIRS of the small bowel reflects changes in blood flow into the small bowel and/or portal venous flow into the liver during IIR in adult rats.

Methods and Materials

Experiment 1: NIRS monitoring of intestinal ischaemia-reperfusion in suckling rats

Animal preparation

Mixed sex Sprague-Dawley suckling rats (11 – 13 days old), median weight 22g (range 15 – 25g) were studied. Rats were kept with their dams and allowed to suckle freely until anaesthetised. The family group was kept under standardised conditions for light and temperature. Dams were kept under standardised conditions for food and water and allowed access to standard rat chow and water *ad libitum*. Animal numbers reported below refer to animals completing the entire experimental protocol.

There were three experimental groups:

- A) Sham operation for 105 minutes (n=3)
- B) Intestinal ischaemia only for 105 minutes (n=3)

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C) Intestinal ischaemia-reperfusion (20 minutes 'run-in' + 45 minutes ischaemia + 60 minutes reperfusion (n=4)

Rectal temperature was monitored continuously immediately after the induction of deep surgical anaesthesia (absence of response to firm web-space pinch) and maintained at $37 \pm 0.5^\circ\text{C}$ using a heating pad and radiant lamp.

Surgical procedure

All animals were anaesthetised with 3% halothane in a 50:50 mixture of oxygen and nitrous oxide at a flow rate of 500ml/min per gas, until deep surgical anaesthesia had been induced. Anaesthesia was maintained using 0.8-1.5% halothane in a 50:50 mixture of oxygen and nitrous oxide at the same flow rates for the remainder of the experiment. All animals had a transverse laparotomy and exposure of the SMA. Groups B and C had a 3/0 silk tie placed loosely under the SMA to aid relocation. The bladder was decompressed by direct puncture and drained using a 1ml Plastipak® syringe and 21G Microlance®3 sterile needles (needles and syringes from Becton Dickinson UK Ltd, Cowley, Oxford, UK) to ensure that it did not invade the NIRS field. Two 5mm NIRS optodes (NIRO 500, Hamamatsu Photonics UK Ltd., Enfield, UK) were positioned inside the abdominal cavity below the stomach so that near infrared light at four wavelengths passed through small bowel into the detector optode (see figure 6.1).

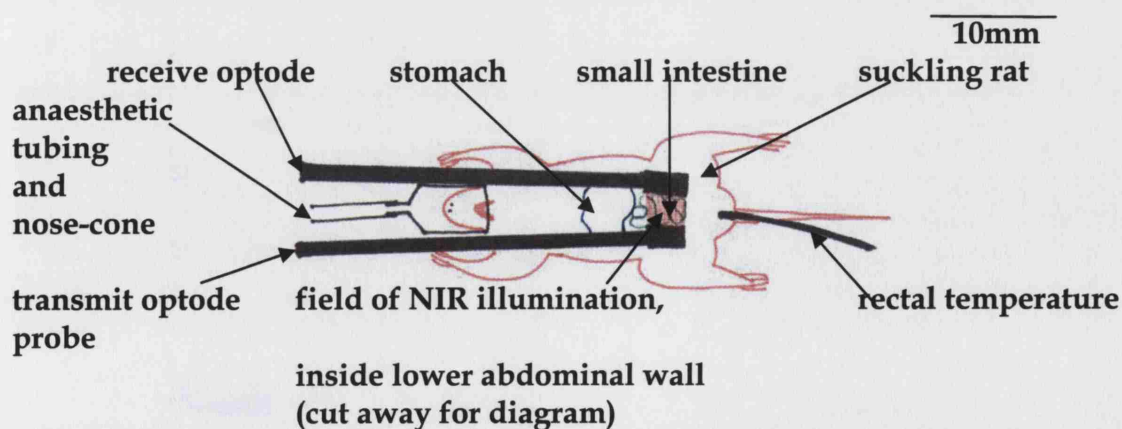


Figure 6.1: Diagram of the positioning of two 5mm NIRS optodes inside the abdominal cavity of a suckling rat (bird's eye view)

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NIR spectra were recorded using a NIRO 500 near infrared spectrometer (Hamamatsu Photonics UK Ltd., Enfield, UK). NIRS readings of oxygenated haemoglobin (HbO) and deoxygenated haemoglobin (Hb) were made every 0.5 seconds, and mean output was recorded every second. The abdomen was shielded from ambient light with an aluminium foil cover and an overlaid swab. When a satisfactory NIRS signal had been established, recording was started.

Group A (sham operation) had no further surgical intervention and NIRS of the small bowel was recorded for 125 minutes. Groups B and C (intestinal ischaemia only [IO] and IIR) had a 20 minute NIRS stabilisation period ('run-in'), then the SMA was located using the previously sited silk sling with minimal displacement of the optodes. A small atraumatic sprung clip was used to occlude the SMA, inducing intestinal ischaemia. Group B had no further surgical intervention and NIRS of the small bowel was recorded for a further 105 minutes. Group C had 45 minutes of intestinal ischaemia and NIRS of the small bowel, then the clip was removed without disturbing the NIRS optodes and NIRS recording continued for a further 60 minutes.

Data processing

NIRS digital output was processed using custom written software (NirSpec, Biomedical Engineering Department, Great Ormond Street Hospital for Children). Data was exported into Microsoft Excel 2002 (Microsoft Office XP Professional with Publisher version 2002, Microsoft Corporation, Redmond, Washington, USA) for display and analysis. HbO and Hb concentrations were rescaled to correct for negative values obtained (because all readings were made relative to initial values recorded) and 60-second mean values calculated. Tissue oxygenation index (TOI) was calculated according the following formula:

$$\text{TOI} = \text{HbO} / (\text{HbO} + \text{Hb})$$

where: TOI denotes tissue oxygenation index

HbO denotes corrected oxyhaemoglobin concentration

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Hb denotes corrected deoxyhaemoglobin concentration

Statistical analysis

Data for experiment 1 are presented as simultaneous plots of individual animals' 60-second mean TOI over experimental time for each treatment group (sham operation, IO and IIR), and as separate plots of group data of 60-second group mean \pm standard error of the mean (SEM) over experimental time for animals of the same treatment group. Statistical analysis was performed by comparison of summary measures by analysis of variance after testing for normality of data. The summary measurements used in this experiment were the areas under the TOI curve during the three experimental phases ('run-in' [1st - 20th minute], intestinal ischaemia [21st - 65th minute] and intestinal reperfusion [66th - 125th minute], or equivalent time periods in the sham and intestinal ischaemia only groups), and over the whole experiment. Correction for multiple comparisons adjusts the *P* value indicating significant differences to less than 0.0125. All statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, CA, USA).

Experiment 2: NIRS and blood flow monitoring during ischaemia-reperfusion of the small bowel in adult rats

Animal preparation

Age-matched adult male Sprague-Dawley rats (8 – 12 weeks old) weighing between 246g and 298g (median weight was 270g) were studied. Rats were kept under standardised conditions for food, water, light and temperature. All animals had access to standard rat chow and water *ad libitum*. Animal numbers reported below refer to animals completing the entire experimental protocol.

There were two experimental groups:

- A) Sham operation for 180 minutes (n=9)

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B) IIR (30 minutes run-in + 90 minutes ischaemia + 60 minutes reperfusion) (n=11)

Rectal temperature was monitored continuously immediately after induction of deep surgical anaesthesia (absence of response to firm web-space pinch), and maintained at $37 \pm 0.5^{\circ}\text{C}$ using a heating pad and radiant lamp.

Surgical procedure

Animals were anaesthetised with 3% halothane by inhalation in a 50:50 mixture of oxygen and nitrous oxide at a flow rate of 200ml/min per gas until deep surgical anaesthesia had been induced. Anaesthesia was maintained using 0.8-1.5% halothane in a 50:50 mixture of oxygen and nitrous oxide at the same flow rates for the remainder of the experiment. An arterial line was positioned in the right common femoral artery and flushed with 0.5ml of heparinised saline (25 units lithium heparin salt (Sigma Chemical Co, St Louis, MO, USA) per ml). After calibration with a sphygmomanometer, the arterial line was used to measure arterial blood pressure via a transducer (TranspakIV Abbott Pressure Transducer, Abbott Laboratories, Chicago, IL, USA) connected to the same Transonic® flowmeter (T206 Transonic® Animal Research Flowmeter, Transonic Systems Inc., Ithaca, NY, USA) used to record flow in the portal vein and SMA. All animals had midline laparotomy and exposure of the SMA. A 1.5mm R Transonic® transit-time ultrasound (TTUS) flow probe (Transonic Systems Inc., Ithaca, NY, USA) was sited around the portal vein such that flow was not restricted by the probe. A 0.7mm V Transonic® TTUS flow probe (Transonic Systems Inc., Ithaca, NY, USA) was sited around the SMA and adjusted to give an acceptable trace without restricting flow in the artery. Acoustic coupling within the flow probes was facilitated by application of water-based sterile jelly (H.R. Lubricating jelly, Carter Products, Division of Carter-Wallace, Inc., New York, NY, USA) around the blood vessels. Flow and blood pressure were measured using a T206 Transonic® Animal Research Flowmeter (Transonic Systems Inc., Ithaca, NY, USA).

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Two NIRS optodes (Schott Fibre Optics (UK) Ltd., Doncaster, UK) were mounted on a small polystyrene block and balanced over several loops of small bowel (see figure 6.2).

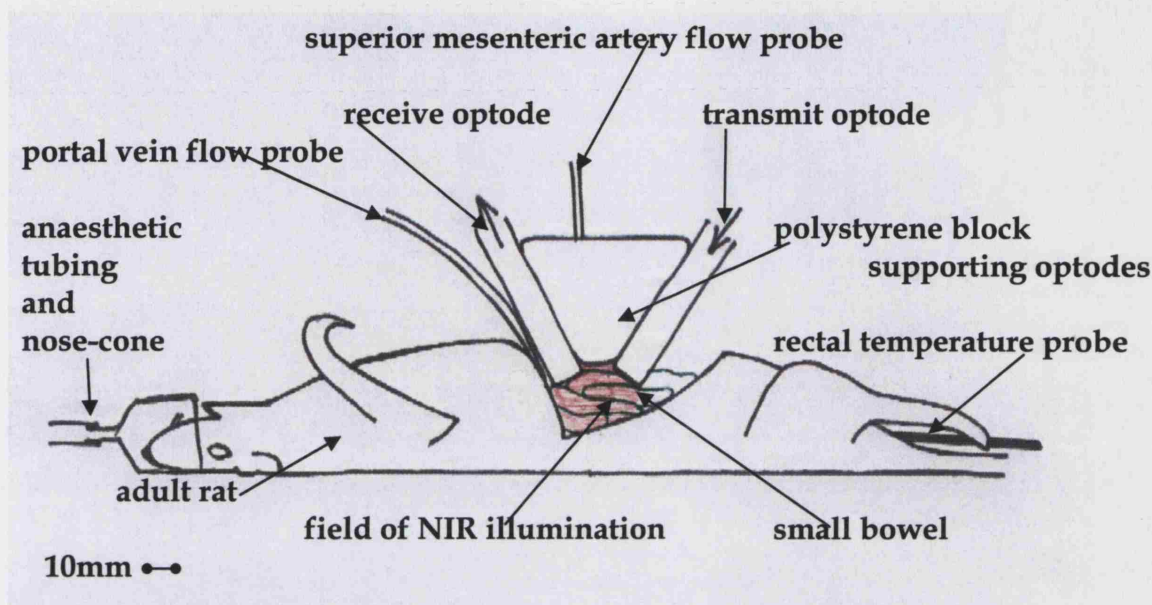


Figure 6.2: Diagram of the positioning of NIRS optodes, flow probes and other monitoring during IIR and sham operation in adult rats (lateral view).

The optodes were in contact with the bowel, but not pressing on it (no bowel deformation was visible at any time). NIR light was produced by a 77501 DC regulated quartz halogen light source (Oriol Instruments, UK) using high and low pass filters (Melles Griot, CA, USA) to constrain delivered wavelengths to the range 650 – 950nm. NIR light was transmitted into the abdomen by one optode, passed predominantly through small bowel and was detected by the second optode. Detected NIR light was transmitted to a 270 M continuous spectrum NIR spectrometer (Instruments SA, UK) in serial communication with a 5-stage, air-cooled charge-coupled device (CCD) 30-11 camera (Wright Instruments Ltd, UK). Spectra were recorded continuously and averaged over 1009ms per output data-point. Ambient light was reduced with a grey felt screen, and the abdomen was partially covered with a damp swab to reduce evaporative fluid loss.

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When satisfactory signals had been established from portal vein and SMA flow probes, the blood pressure transducer and the NIRS optodes, recording was started. Set-up time was between 25 and 60 minutes (median time was 35 minutes). Group A (sham operation) had no further surgical intervention and recording was continued for 180 minutes. Group B (IIR) had 30 minutes of 'run-in' without surgical intervention. When group B had completed the 'run-in' period, the flow probe recording SMA flow was raised without disturbing the position of any of the other recording devices. Flow in the SMA was obstructed and intestinal ischaemia confirmed by direct observation of absence of pulsation in small bowel mesenteric arteries. Recording was continued for 90 minutes of intestinal ischaemia, then the SMA flow probe was lowered without disturbing any of the other recording devices, and flow was permitted again in the SMA. Reperfusion of the intestine was confirmed by direct observation of the return of pulsatile flow in the arteries of the small bowel mesentery. Recording was continued for 60 minutes of intestinal reperfusion.

During the first 20 minutes of recording a brief (less than 60 seconds) anoxic insult (1% halothane in 100% nitrous oxide) was given to selected animals in both groups. This caused all HbO in the animal to desaturate resulting in the conversion of all HbO into Hb. This achieved a true zero NIRS reading for absolute HbO concentration (NIRS recording always begins at an arbitrary level) which could be used during processing to adjust recordings to give absolute concentrations of HbO (Quaresima et al, 1998). After a short period of respiratory arrest (less than 10 seconds) mechanical resuscitation was started (a maximum of 20 seconds of chest compressions) and animals made a good recovery within 5 minutes of the hypoxic insult and completed the experimental protocol (with the exception of a single animal which failed to respond to resuscitation and died).

Data processing

Blood pressure and flow were recorded using WinDaq software (Dataq Instruments, Akron, Ohio, USA) at a sample rate of 100 data points per second. Advanced Cudas software (Dataq Instruments, Akron, Ohio, USA) was used to select peaks in the blood pressure waveform to calculate heart rate. NIRS digital output was processed using custom written software (Tarragona, Department of Medical Physics and Bioengineering, University College London, UK) supplied by Dr Roger Springett. HbO concentration was estimated directly from the NIR spectrum using one custom-written algorithm (Dr Roger Springett, Tarragona, Department of Medical Physics and Bioengineering, University College London, UK). Hb concentration was derived from the second differential of the water signal, which was also used to calculate path-length, using a separate custom-written algorithm (Dr Roger Springett, Tarragona, Department of Medical Physics and Bioengineering, University College London, UK). Zero HbO levels were used to adjust NIRS recordings to reflect absolute HbO concentration. Animals without a measured zero HbO level were assigned an estimated value derived from the values obtained experimentally in other animals. Absolute Hb concentration did not require individual adjustment. TOI was calculated according the following formula:

$$\text{TOI} = \text{HbO} / (\text{HbO} + \text{Hb})$$

where: TOI denotes tissue oxygenation index

HbO denotes absolute oxyhaemoglobin concentration

Hb denotes absolute deoxyhaemoglobin concentration

Data were exported into Microsoft Excel 2002 (Microsoft Office XP Professional with Publisher version 2002, Microsoft Corporation, Redmond, Washington, USA) for time-matching and analysis.

Statistical analysis

Data are presented in two sections for experiment 2. Firstly, three representative whole experimental recordings [sham operation, IIR (1) and IIR (2)] from individual animals are shown as simultaneous plots of 60-second mean values for all parameters measured over experimental time. Secondly, group data for animals of the same experimental group (sham operation or IIR) are shown as separate plots of 60-second group mean \pm SEM over experimental time for heart rate, blood pressure, blood flow (portal venous flow [PVF] and SMA flow [SMAF]), small bowel HbO concentration, small bowel Hb concentration and small bowel TOI over experimental time. Statistical analysis of group data was performed by comparison of summary measures for each parameter measured. Summary measurements selected for statistical analysis were group mean values during the 30th (last minute of run-in), 32nd (first full minute of intestinal ischaemia), 120th (last full minute of intestinal ischaemia), 122nd (first full minute of intestinal reperfusion) and 180th minute (last full minute of intestinal reperfusion). After testing for normality of data, repeated measures ANOVA with Tukey's multiple post-hoc comparisons or test for linear trend over time was used for within experimental group comparisons, and unpaired t-tests with Bonferroni correction for multiple measurements for between experimental group comparisons. All statistical analysis was performed using GraphPad Prism software (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, CA, USA).

Also presented are simultaneous plots of group data of small bowel TOI and SMAF or PVF over experimental time for both sham operation and IIR groups, and the ratio of SMAF or PVF to TOI over experimental time for both sham operation and IIR groups.

Results

Experiment 1: NIRS monitoring of intestinal ischaemia-reperfusion in suckling rats

Figure 6.3 shows TOI during sham operation over experimental time in suckling rats.

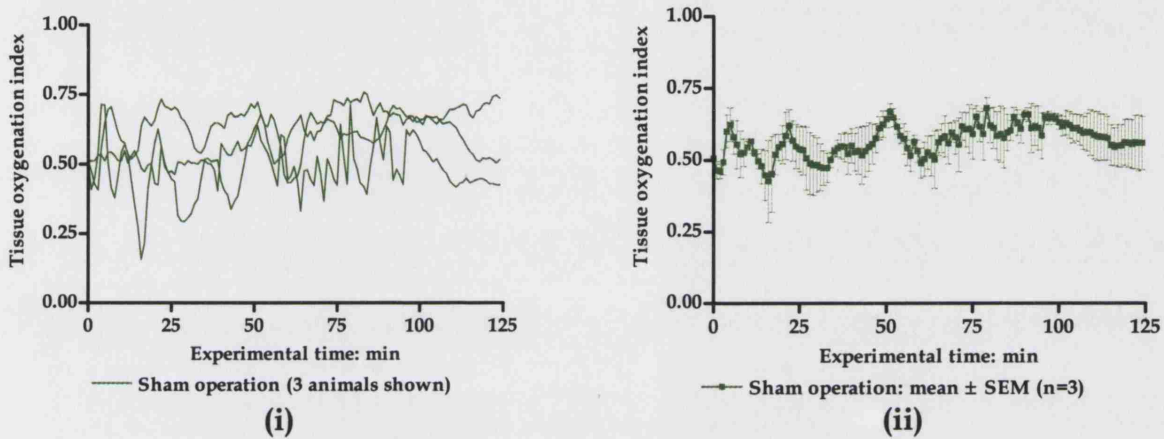


Figure 6.3: TOI of suckling rat gut during sham operation:
(i) individual animal recordings (60-second mean values)
(ii) group mean \pm SEM (60-second group mean values)

Figure 6.4 shows TOI during IO over experimental time in suckling rats.

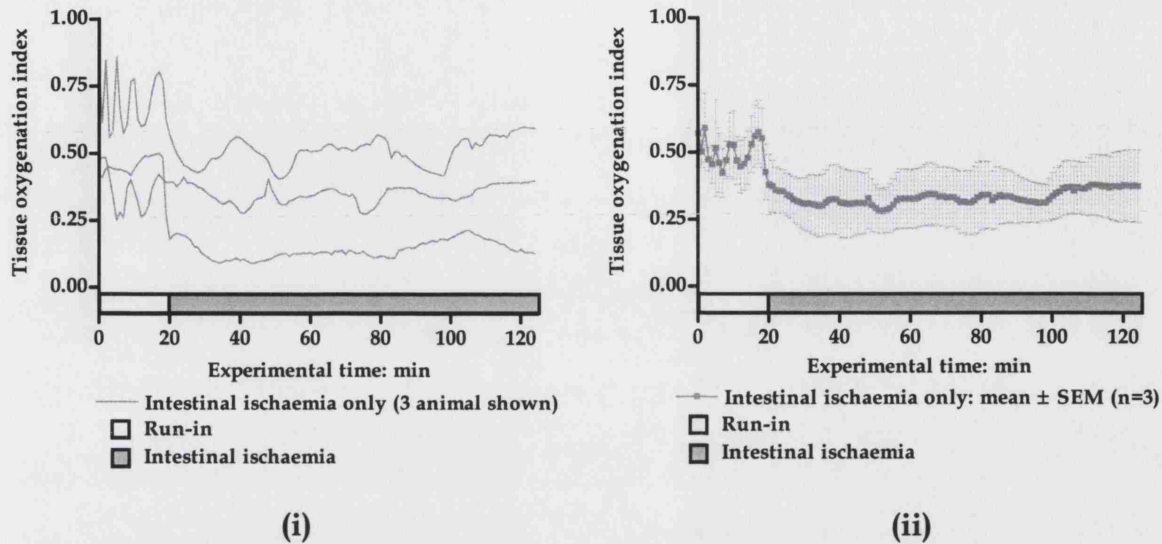


Figure 6.4: TOI of suckling rat gut during IO:
 (i) individual animal recordings (60-second mean values)
 (ii) group mean \pm SEM (60-second group mean values)

Figure 6.5 shows TOI during IIR over experimental time in suckling rats.

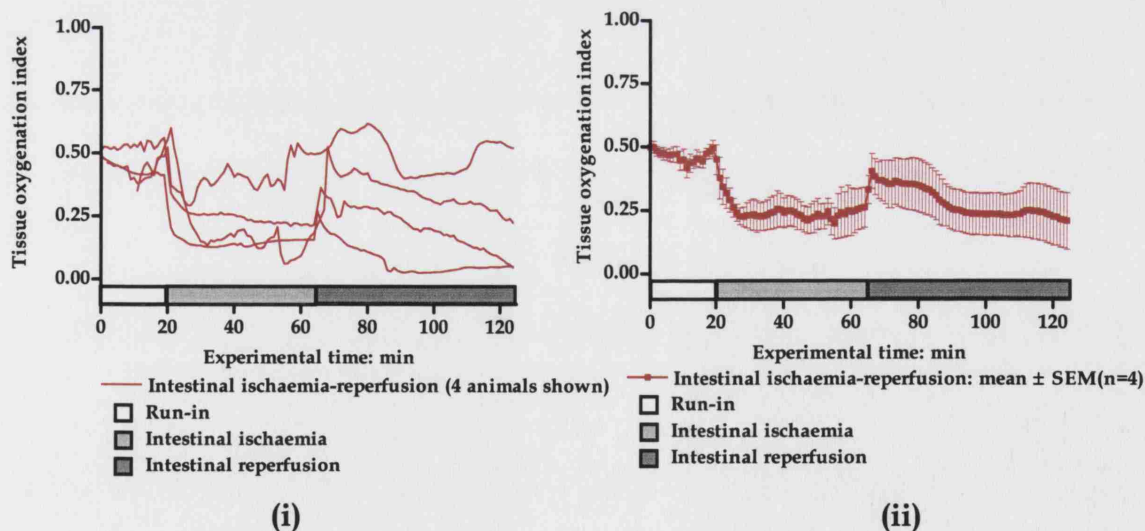


Figure 6.5: TOI of suckling rat gut during IIR:
 (i) individual animal recordings (60-second mean values)
 (ii) group mean \pm SEM (60-second group mean values)

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Mean TOI levels were approximately 0.5 in the sham group throughout the experiment, with marked intra- and inter-animal variation. A similar pattern was seen in mean TOI levels of the IO group during 'run-in', but during intestinal ischaemia TOI fell in two out of three animals, intra-animal variability decreased and the group mean TOI was closer to 0.3 for the remainder of the experiment. IIR animals showed much less intra-animal variability than the sham group. All IIR animals showed a drop in TOI with intestinal ischaemia that was maintained throughout ischaemia in 3 out of 4 animals. Intestinal reperfusion was associated with a recovery in TOI in 3 out of 4 animals, but this was not maintained during prolonged reperfusion. Mean TOI levels were approximately 0.5 and 0.25 during 'run-in' and intestinal ischaemia respectively, but declined from 0.4 to 0.2 over the 60 minutes of intestinal reperfusion.

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Summary measurements for this pilot study in suckling rats are shown in figure 6.6.

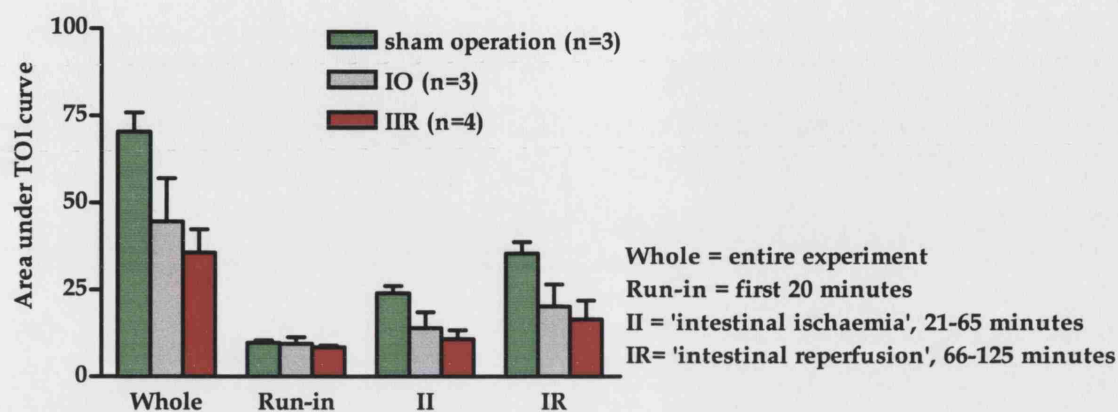


Figure 6.6: Comparison of area under TOI curve for suckling rats after sham operation, IO or IIR

ANOVA, between treatment groups:

Whole experiment: $P = 0.05$

Run-in, first 20 minutes: $P = 0.69$

'Intestinal ischaemia', 21-65 minutes: $P = 0.05$

'Intestinal reperfusion', 66-125 minutes: $P = 0.09$

[NB After correction for multiple comparisons, $P < 0.0125$ is accepted as significant]

Comparison of the summary measurements selected for this experiment (area under the TOI curve for each animal in each of the three phases of the intestinal ischaemia-reperfusion group treatment, and the total area under the TOI curve) did not yield any statistically significant differences. However the sham group did appear to maintain a higher TOI than either of the other groups, which approached statistical significance when examined for the experiment as a whole, as well as during intestinal ischaemia and intestinal reperfusion.

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Experiment 2: NIRS and Blood flow monitoring during intestinal ischaemia-reperfusion in adult rats

Figure 6.7 shows a representative example of a whole experimental record for an individual adult rat in the sham operation group.

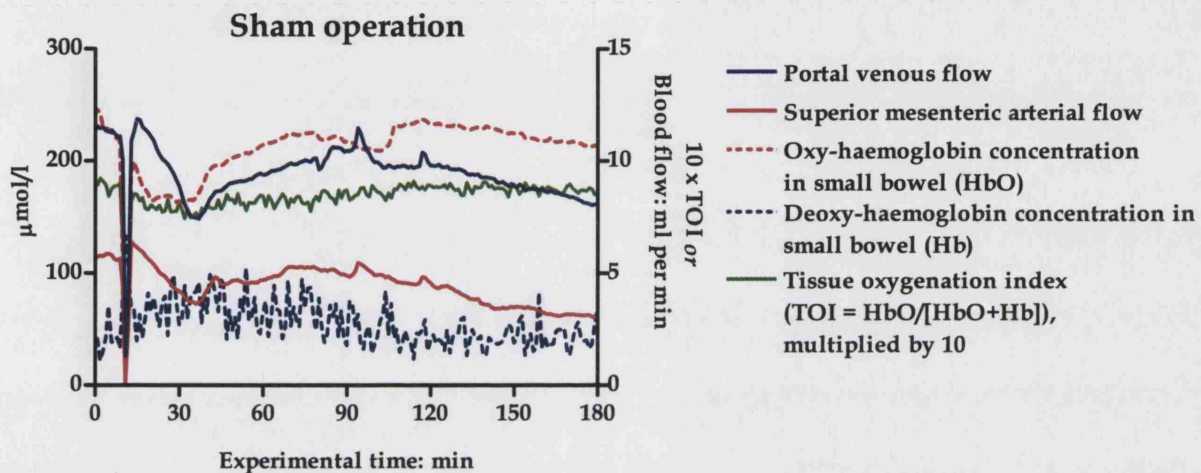


Figure 6.7: Example of a whole experimental recording from an adult rat in the sham operation group

The effect of the brief hypoxic insult at 10 minutes is clearly seen in figure 6.7 (sham operation group). Note that Hb variability is greater than HbO variability throughout the experiment, and that TOI is maintained at approximately 0.8 for the whole experiment (aside from the hypoxic insult).

Figure 6.8 shows a representative example of a whole experimental record for an individual adult rat in the IIR (A) group.

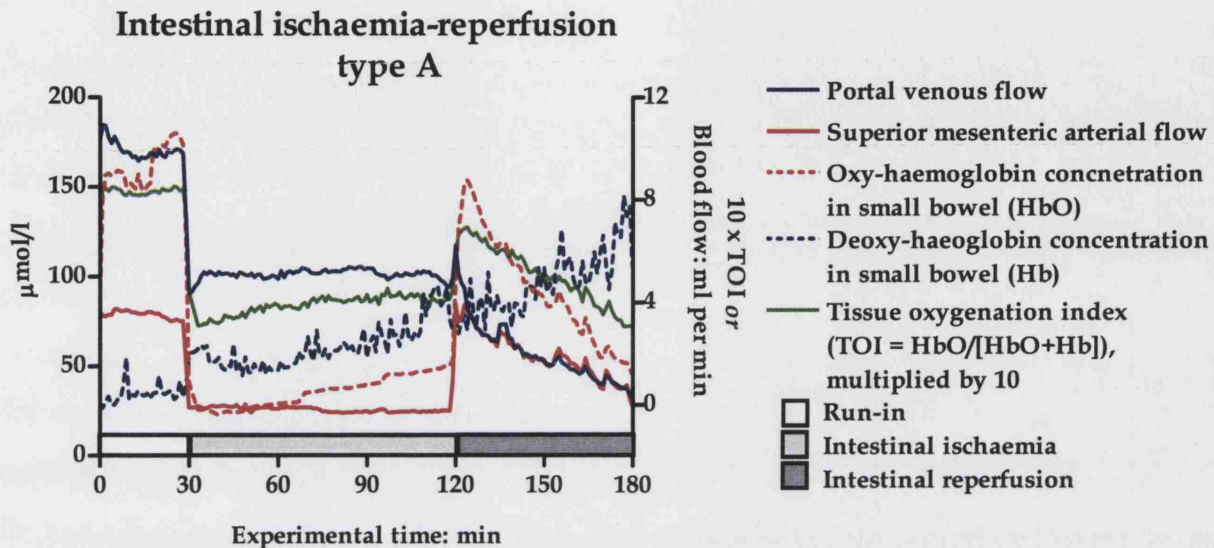


Figure 6.8: Example of a whole experimental recording from an adult rat in the IIR (A) group: note the decline in TOI during intestinal reperfusion.

Figure 6.9 shows a representative example of a whole experimental record for an individual adult rat in the IIR (B) group.

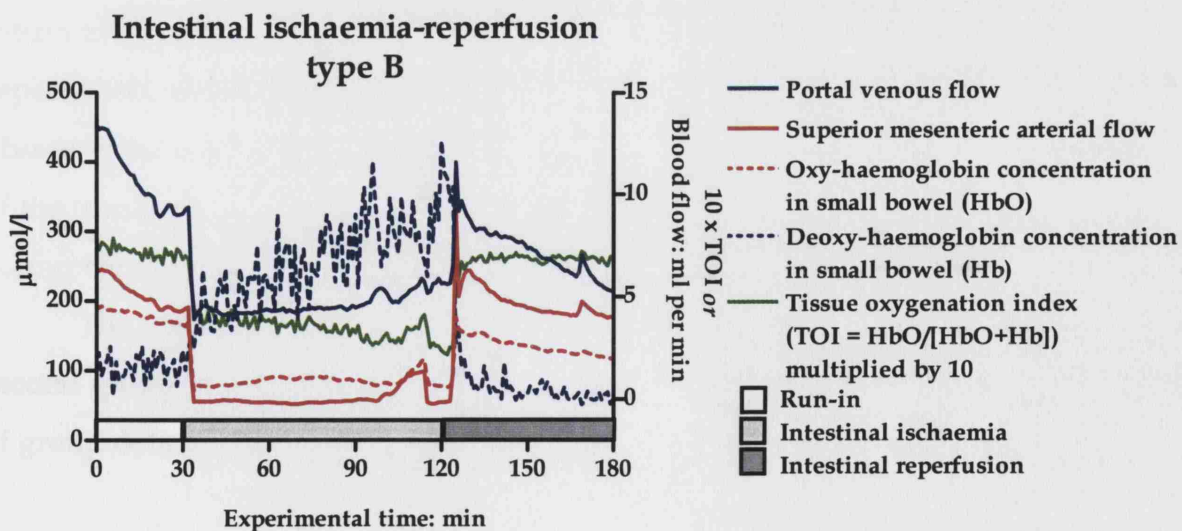


Figure 6.9: Example of a whole experimental recording from an adult rat in the IIR (B) group: note that TOI is sustained throughout intestinal reperfusion.

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Two IIR traces are shown (figures 6.8 and 6.9), as the IIR animals fell into two broad groups based on recorded observations during intestinal reperfusion. The first trace (figure 6.8) shows a typical response to 'run-in' (as per sham group) followed by a rapid drop in flow in SMA and portal vein with intestinal ischaemia. HbO concentration and TOI also fall steeply with intestinal ischaemia. Hb concentration shows a slow rise throughout intestinal ischaemia. Intestinal reperfusion results in a rapid recovery of flow in the SMA and portal vein with concomitant increases in TOI and HbO. In these animals [IIR (A)], SMAF, PVF, TOI and HbO levels all decline throughout intestinal reperfusion to reach levels approaching those seen during intestinal ischaemia by the end of the experiment. Hb continues to rise throughout intestinal reperfusion, achieving levels higher than those seen during intestinal ischaemia. Seven out of eleven IIR animals behaved in this way. The second group of IIR animals [IIR (B)] showed a different pattern of changes during intestinal reperfusion (figure 6.9). Levels of SMAF, PVF, TOI and HbO achieved on initial intestinal reperfusion are largely maintained for the rest of the experiment, and are similar to levels seen during 'run-in'. Hb levels return to levels seen during 'run-in,' instead of rising throughout intestinal reperfusion. Four out of eleven IIR animals behaved in this way. As these observations were made after the experiment had been completed, the remainder of the results displayed below do **not** take this subdivision of the IIR group into account, and include data from all 11 animals treated with IIR.

The rest of the data shown for experiment 2 are presented as group data (60-second group mean \pm SEM) over experimental time, and summary measurements of group data (60-second group mean for selected time-points).

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Figure 6.10 shows group mean heart rate over experimental time during sham operation or IIR in adult rats.

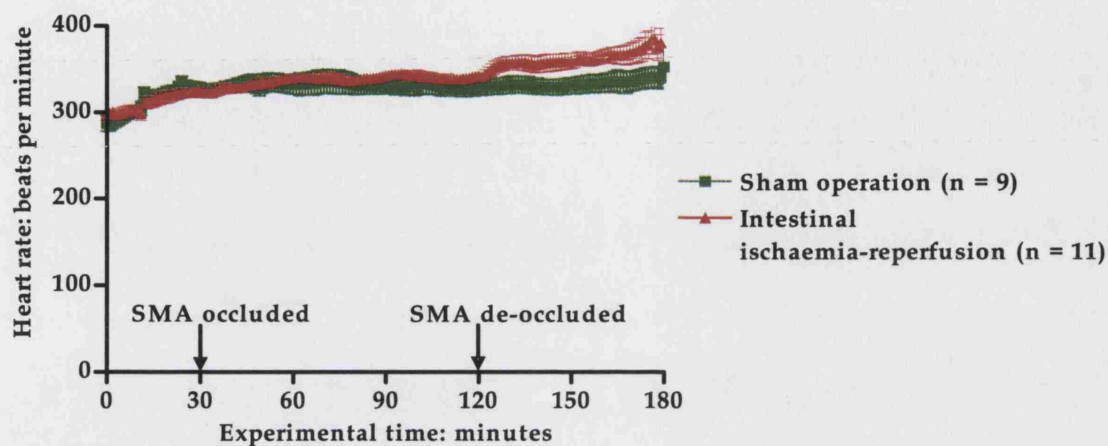


Figure 6.10: Heart rate during sham operation or IIR in adult rats: group data (mean \pm SEM)

Heart rate rose during both IIR and sham operation during run-in. Sham animals subsequently maintained approximately the same mean heart rate for the remainder of the experiment. Heart rate gradually increased in animals undergoing IIR during intestinal reperfusion.

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Summary measurements of heart rate group data are shown in figure 6.11.

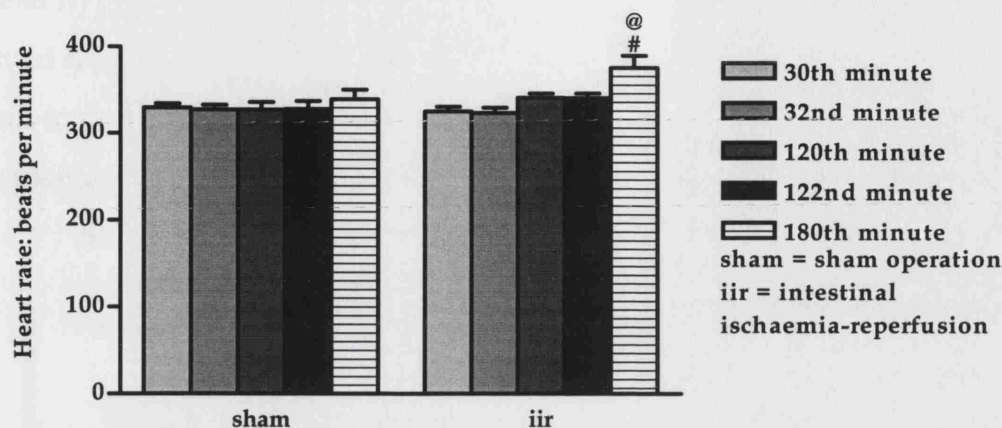


Figure 6.11: Summary measurements of group data for heart rate during sham operation or IIR in adult rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

A small but significant rise in heart rate was seen in the 180th minute after IIR only.

Figure 6.12 shows group mean MAP (mean arterial blood pressure) over experimental time during sham operation or IIR in adult rats.

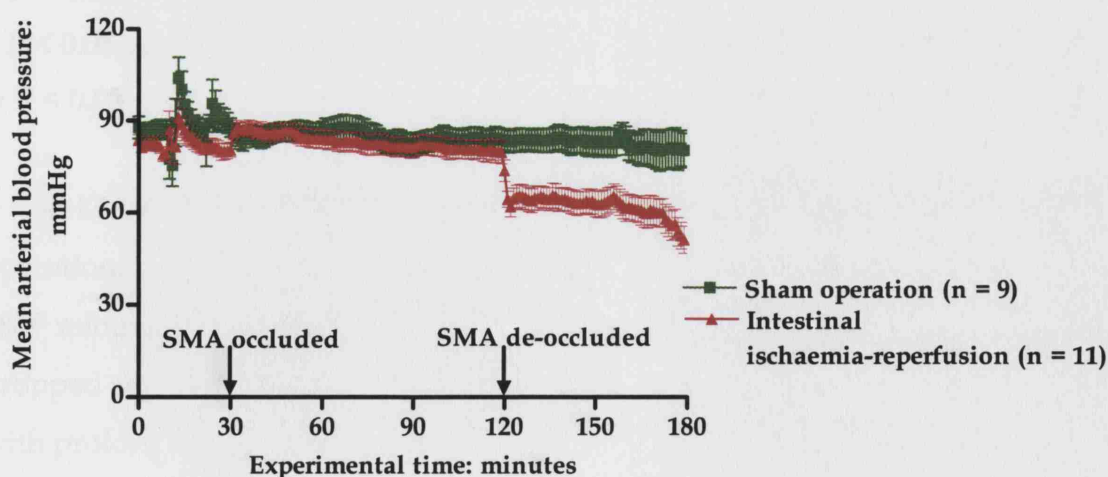


Figure 6.12: MAP during sham operation or IIR in adult rats: group data (mean \pm SEM)

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MAP in the sham group declined slowly throughout the experiment. The brief hypoxic insult given at 10 minutes to calibrate NIRS readings caused a short-lived rise in MAP during the recovery period; this had resolved by the end of the run-in period. MAP dropped in the IIR group on intestinal reperfusion and remained low throughout intestinal reperfusion.

Summary measurements of MAP group data are shown in figure 6.13.

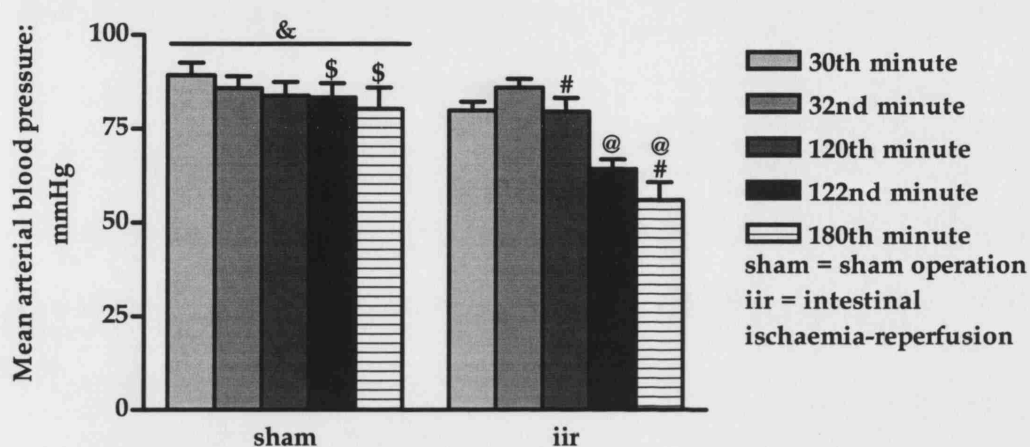


Figure 6.13: Summary measurements of group data for MAP during sham operation or IIR in adult rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus iir, same time point

& $P < 0.05$ (change over experimental time)

MAP declined significantly over the course of the experiment with sham operation, but remained significantly higher than in the IIR group at the 122nd and 180th minutes. In the IIR group, MAP was not affected by intestinal ischaemia but dropped significantly immediately after intestinal reperfusion, and fell further with prolonged reperfusion.

Figure 6.14 shows group mean PVF over experimental time during sham operation or IIR in adult rats.

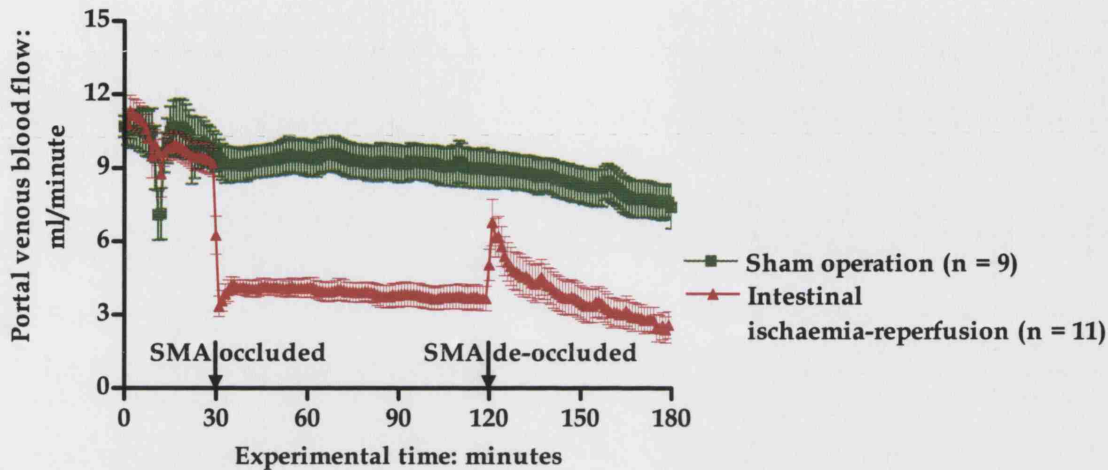


Figure 6.14: PVF during sham operation or IIR in adult rats: group data (mean \pm SEM)

PVF declined over the course of the experiment in the sham group. The effect of the brief hypoxic insult given at 10 minutes during the run-in period is clearly seen, with a transient drop in portal flow followed by a small rebound increase that had largely resolved by the end of the run-in period. In the IIR group, portal venous flow dropped sharply on occlusion of the SMA; recovered on SMA de-occlusion; but then declined with increasing intestinal reperfusion to a level significantly lower than that seen in the sham group at 180 minutes.

Summary measurements of PVF group data are shown in figure 6.15.

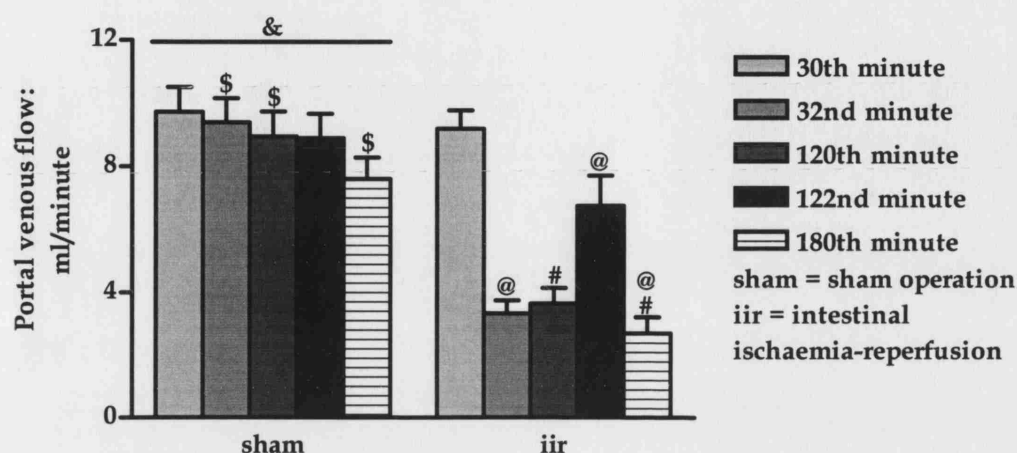


Figure 6.15: Summary measurements of group data for PVF during sham operation or IIR in adult rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus iir, same time point

& $P < 0.05$ (change over experimental time)

PVF in the sham operation group fell significantly over experimental time. With IIR, PVF fell significantly with SMA occlusion (to a level below that of the sham group at the same time-point and below PVF at the end of run-in in the IIR group). This low flow was maintained throughout intestinal ischaemia. On SMA de-occlusion, PVF recovered in the IIR group to the same flow rate seen in the sham group at the same time-point. With prolonged reperfusion, a secondary fall in PVF was seen, with final PVF in the IIR group significantly lower than in the sham group at the same time-point.

Figure 6.16 shows group mean SMAF over experimental time during sham operation or IIR in adult rats.

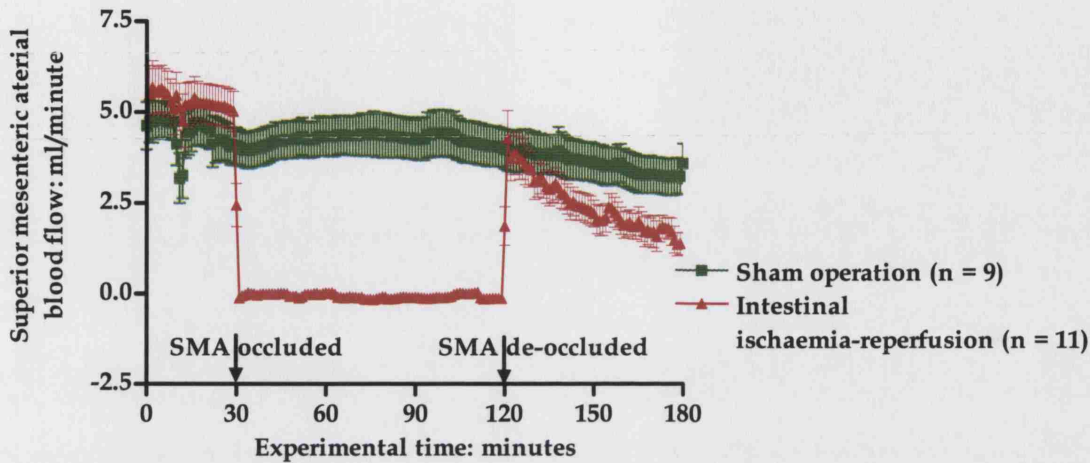


Figure 6.16: SMAF during sham operation or IIR in adult rats: group data (mean \pm SEM)

SMAF followed a broadly similar pattern to that seen in the portal vein in both experimental groups. SMAF declined slightly over the course of the experiment with sham operation, and again the effects of the brief hypoxic insult given at 10 minutes, early in the run-in period are clearly seen. The SMA was successfully occluded in animals in the IIR group to generate intestinal ischaemia. SMAF recovered immediately after SMA de-occlusion, but then declined with prolonged intestinal reperfusion.

Summary measurements of SMAF group data are shown in figure 6.17.

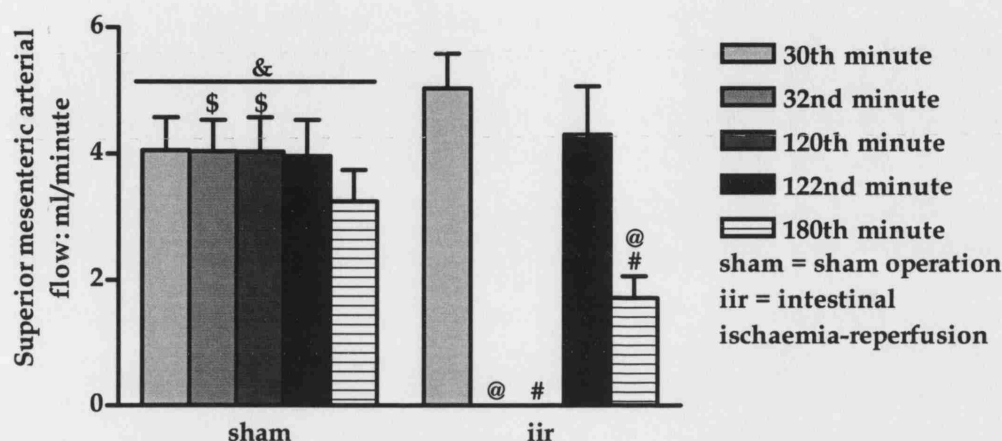


Figure 6.17: Summary measurements of group data for SMAF during sham operation or IIR in adult rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus iir, same time point

& $P < 0.05$ (change over experimental time)

SMAF fell significantly over experimental time in the sham operation group. SMA occlusion in the IIR group resulted in zero flow in the SMA throughout intestinal ischaemia. On SMA de-occlusion, flow in the SMA recovered to a level equivalent to that at the end of run-in in the IIR group, and at the matching time-point in the sham operation group. With prolonged reperfusion, SMAF fell to a level significantly below that seen immediately on reperfusion, and at the end of run-in in the IIR group.

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Figure 6.18 shows group mean small intestinal HbO concentration over experimental time during sham operation or IIR in adult rats.

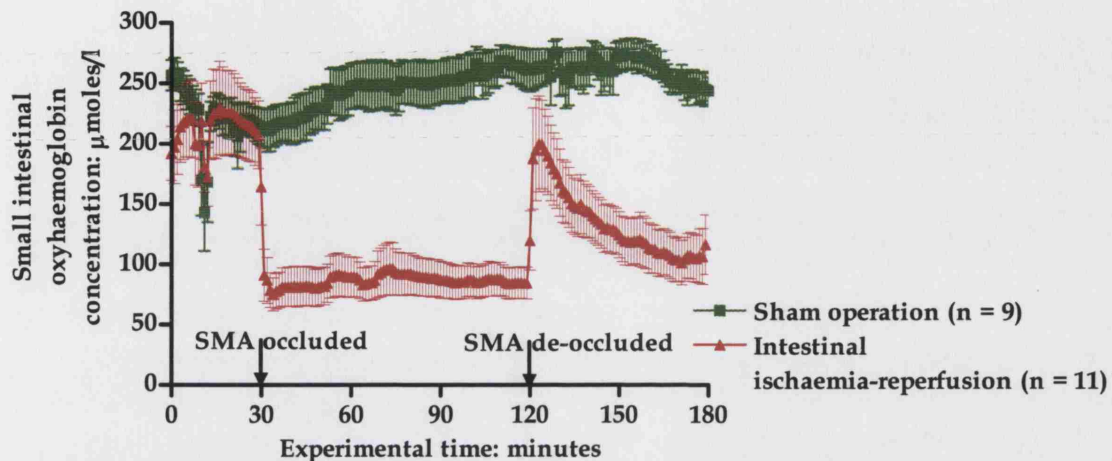


Figure 6.18: Small intestinal HbO concentration during sham operation or IIR in adult rats: group data (mean \pm SEM)

Small intestinal HbO levels echoed the flow patterns seen in the portal vein and SMA in both sham operated and IIR animals with the exception that small intestinal HbO concentration rose slightly throughout the experiment in the sham operation group. Small intestinal HbO concentration fell with SMA occlusion, and recovered with SMA de-occlusion. A decline in small intestinal HbO concentration was seen with prolonged intestinal reperfusion.

Summary measurements of small intestinal HbO concentration group data are shown in figure 6.19.

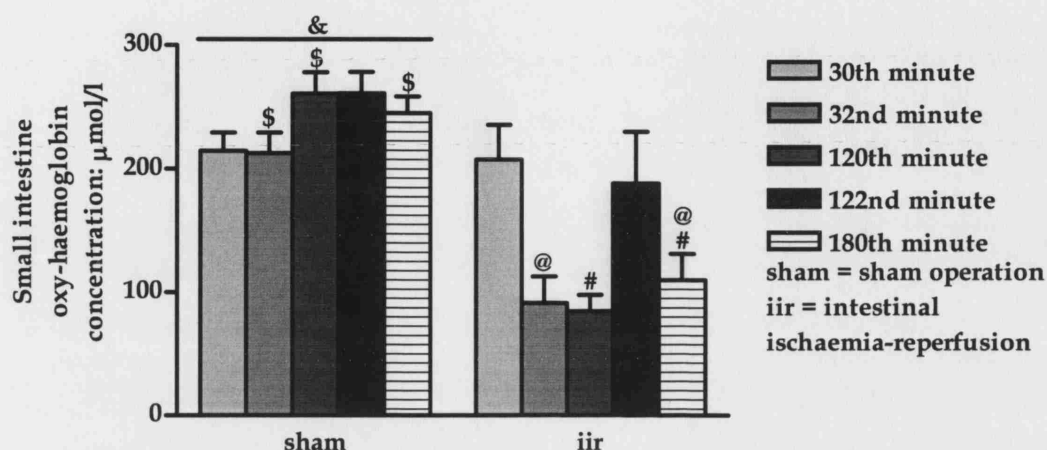


Figure 6.19: Summary measurements of group data for small intestinal HbO concentration during sham operation or IIR in adult rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus iir, same time point

& $P < 0.05$ (change over experimental time)

Small intestinal HbO concentration rose significantly during the experiment in the sham operation group. Small intestinal HbO concentration fell significantly in the IIR group with SMA occlusion (to levels less than IIR run-in and sham operation at matched time-points); recovered to IIR run-in and time-matched sham operation levels with SMA de-occlusion; then fell with prolonged intestinal reperfusion to a level significantly lower than that seen immediately after reperfusion or in the sham group at the end of the experiment.

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Figure 6.20 shows group mean small intestinal Hb concentration over experimental time during sham operation or IIR in adult rats.

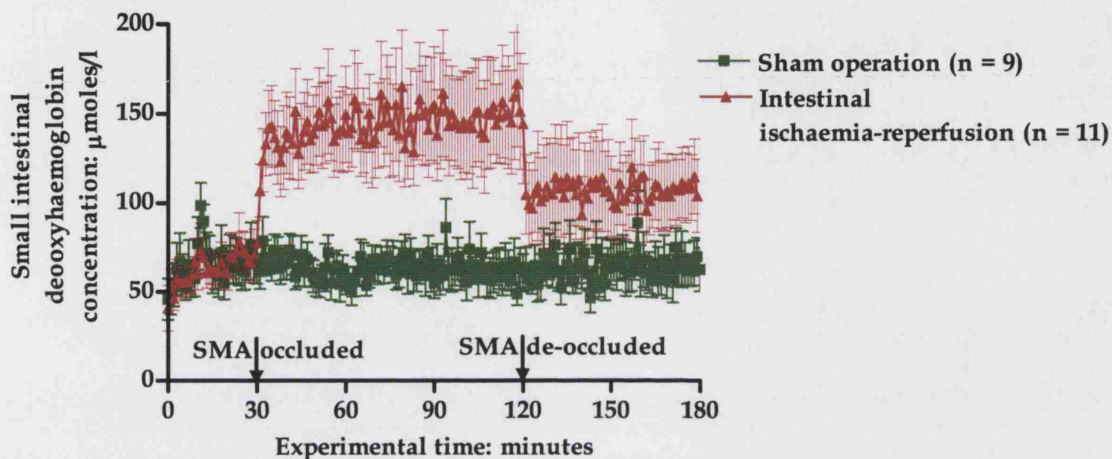


Figure 6.20: Small intestinal Hb concentration during sham operation or IIR in adult rats: group data (mean \pm SEM)

Small intestinal Hb concentration measurements showed much greater intra-animal variability than the other parameters discussed in this chapter. This was related to the derivation of the measurement from the second differential of the water peak at 740nm that introduced greater inherent variability into the measurement. The sham operation group showed no change in small intestinal Hb concentration over experimental time. The IIR group showed a rise in small intestinal Hb concentration with prolonged intestinal ischaemia, and recovery to run-in levels with intestinal reperfusion that were maintained throughout intestinal reperfusion.

Summary measurements of small intestinal Hb concentration group data are shown in figure 6.21.

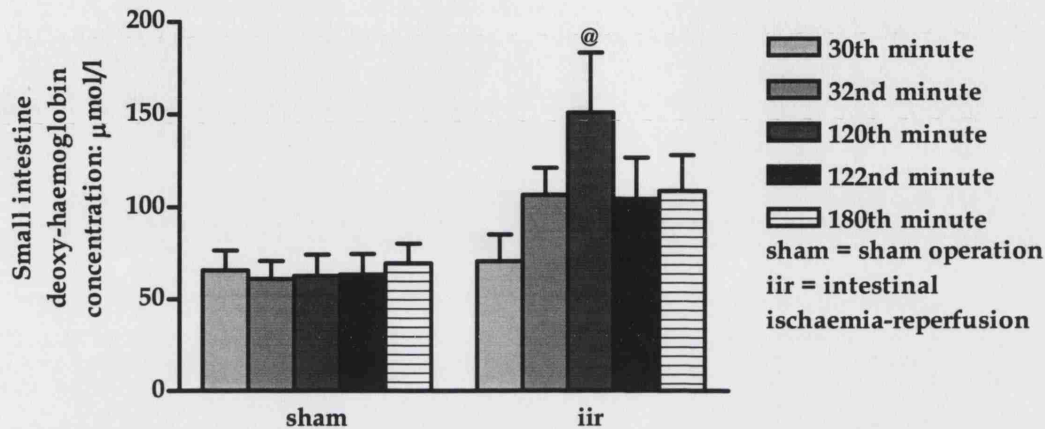


Figure 6.21: Summary measurements of group data for small intestinal Hb concentration during sham operation or IIR in adult rats.

@ $P < 0.05$ versus 30th minute, same treatment

Figure 6.22 shows group mean small intestinal TOI over experimental time during sham operation or IIR in adult rats.

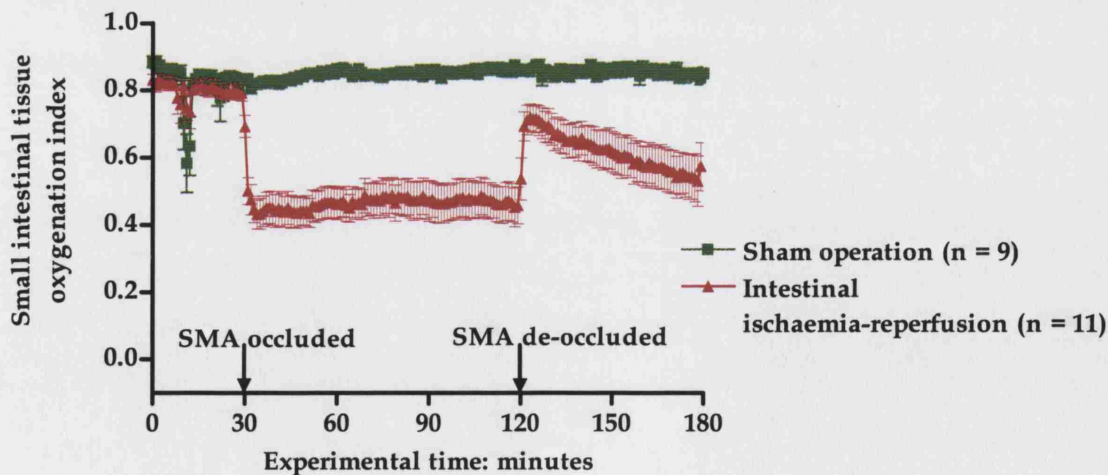


Figure 6.22: Small intestinal TOI during sham operation or IIR in adult rats: group data (mean \pm SEM)

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TOI showed a steady mean level of approximately 0.85 in sham-operated animals after the brief hypoxic insult detailed above. IIR animals exhibited a drop in TOI with intestinal ischaemia, followed by a partial recovery with intestinal reperfusion that was not maintained. As was noted earlier, some IIR animals maintained a high TOI throughout intestinal reperfusion, whereas other IIR animals experienced a fall in TOI with increasing intestinal reperfusion time; this contributes to the widening SEM bars towards the end of intestinal reperfusion. At the end of intestinal reperfusion, mean TOI in the IIR group was significantly lower than that seen in sham operated animals.

Summary measurements of small intestinal TOI group data are shown in figure 6.23.

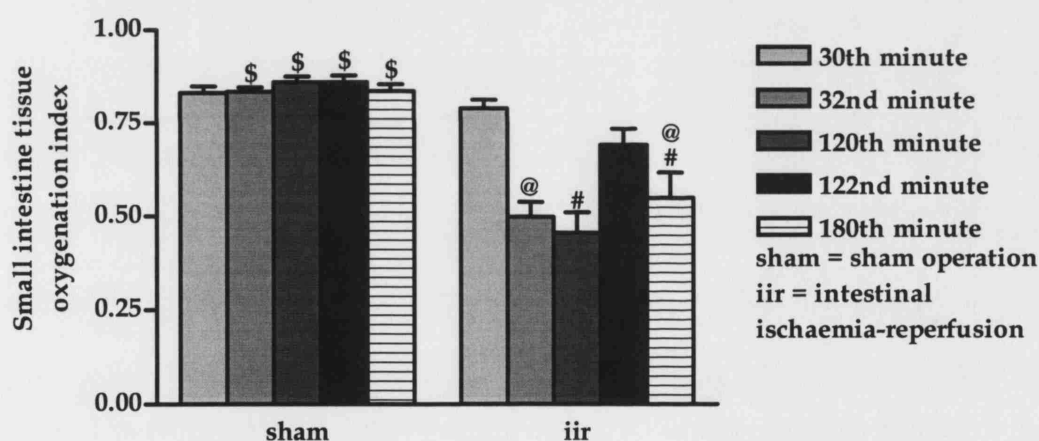


Figure 6.23: Summary measurements of group data for small intestinal TOI during sham operation or IIR in adult rats.

@ $P < 0.05$ versus 30th minute, same treatment

$P < 0.05$ versus 122nd minute, same treatment

\$ $P < 0.05$ versus NT iir, same time point

TOI did not change significantly over experiment time in the sham operation group. TOI fell significantly in the IIR group with intestinal ischaemia, and remained lower than IIR run-in and sham operation levels throughout

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intestinal ischaemia. Immediately on intestinal reperfusion, TOI in the IIR group rose to recover to IIR run-in levels, but remained lower than sham operation levels at a matched time-point. This level of TOI was not maintained in the IIR group, and final TOI was significantly lower than in run-in or on immediate reperfusion in the IIR group, and in the sham operation group.

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The patterns of SMAF and PVF compared to small intestinal TOI in both IIR and sham operated animals was suggestive of a relationship between the two measurements.

Figure 6.24 shows group mean SMAF and small intestinal TOI during IIR over experimental time in adult rats.

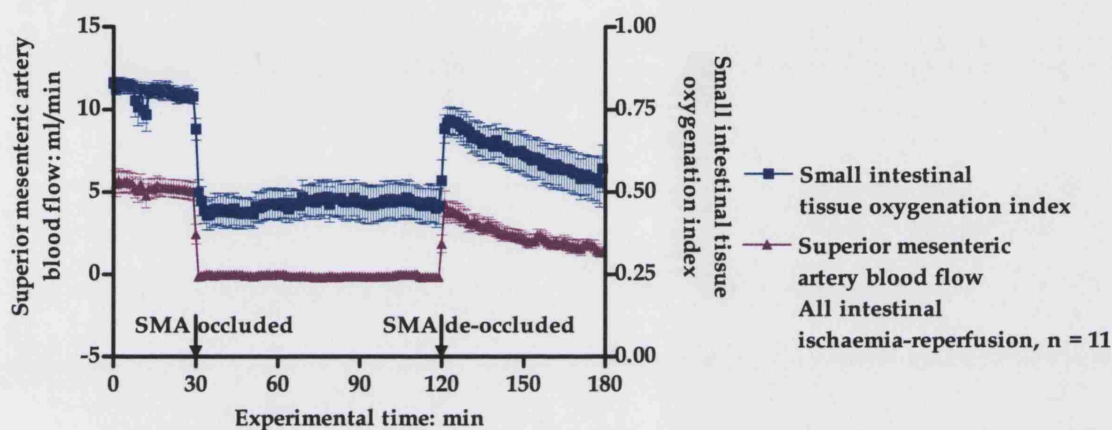


Figure 6.24: SMAF and small intestinal TOI during IIR in adult rats: group mean \pm SEM

Figure 6.25 shows group mean SMAF and small intestinal TOI during sham operation over experimental time in adult rats

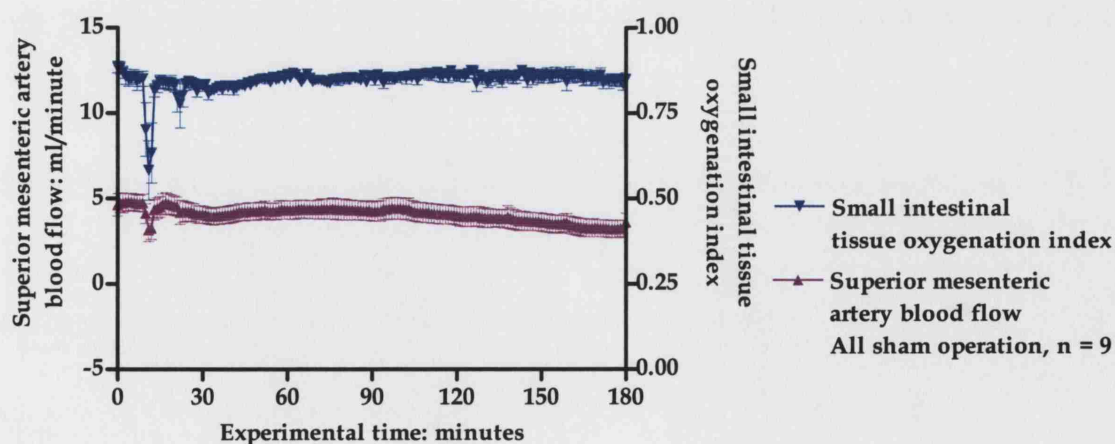


Figure 6.25: SMAF and small intestinal TOI during sham operation in adult rats: group mean \pm SEM

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Figure 6.26 shows group mean PVF and small intestinal TOI during IIR over experimental time in adult rats.

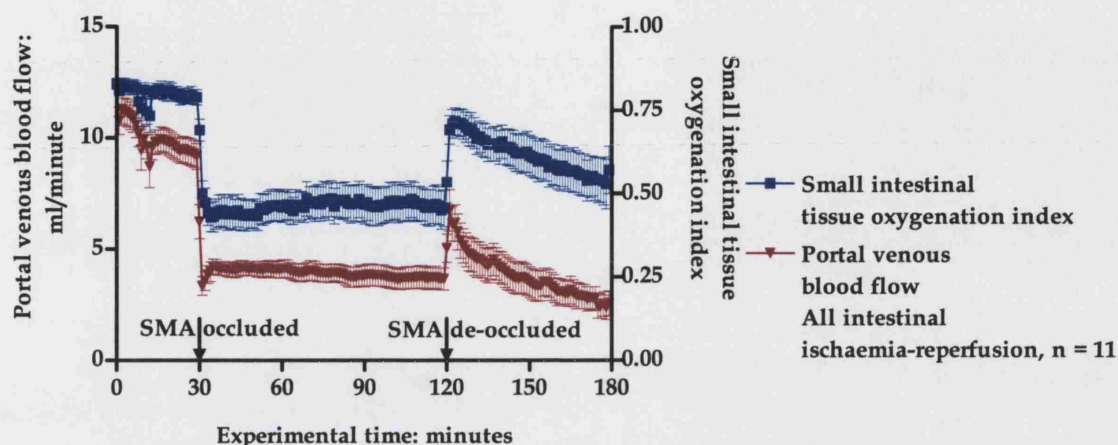


Figure 6.26: PVF and small intestinal TOI during IIR in adult rats: group mean \pm SEM

Figure 6.27 shows group mean PVF and small intestinal TOI during sham operation over experimental time in adult rats.

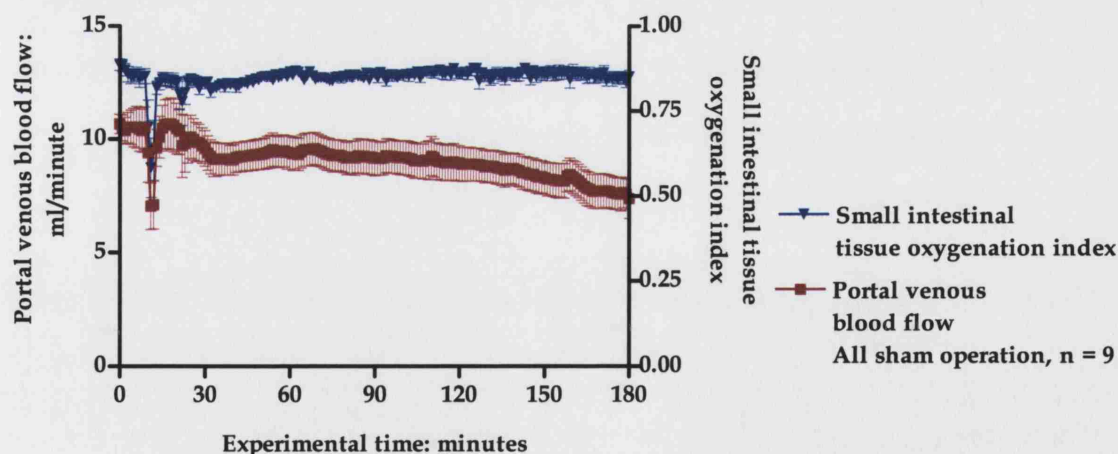


Figure 6.27: PVF and small intestinal TOI during sham operation in adult rats: group mean \pm SEM

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There appears to be a close relationship between both SMA and portal venous blood flow throughout all phases of this experiment. If these parameters are related in a simple way, for example:

$$\text{TOI} \propto (\text{SMAF or PVF})$$

then this relationship could be rewritten as:

$$\text{TOI}/(\text{SMAF or PVF}) = \text{a constant},$$

or: $(\text{SMAF or PVF})/\text{TOI} = \text{a (second) constant}.$

Figure 6.28 shows the ratio of group mean SMAF to TOI during sham operation or IIR over experimental time in adult rats.

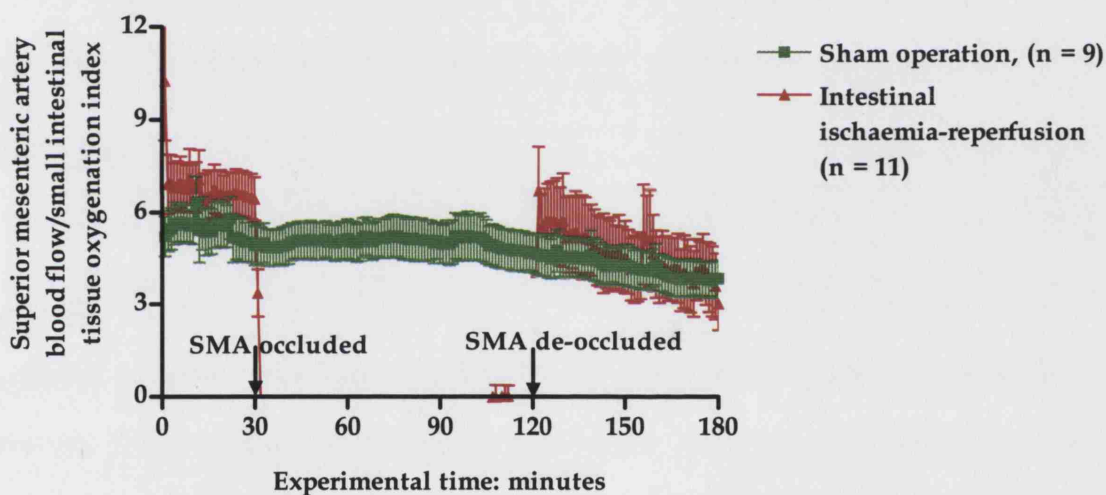


Figure 6.28: Ratio of SMAF to small intestinal TOI during sham operation or IIR in adult rats: group mean \pm SEM.

Note that during cessation of SMA flow during intestinal ischaemia, no ratio is generated for the IIR group.

Figure 6.29 shows the ratio of group mean PVF to TOI during sham operation or IIR over experimental time in adult rats.

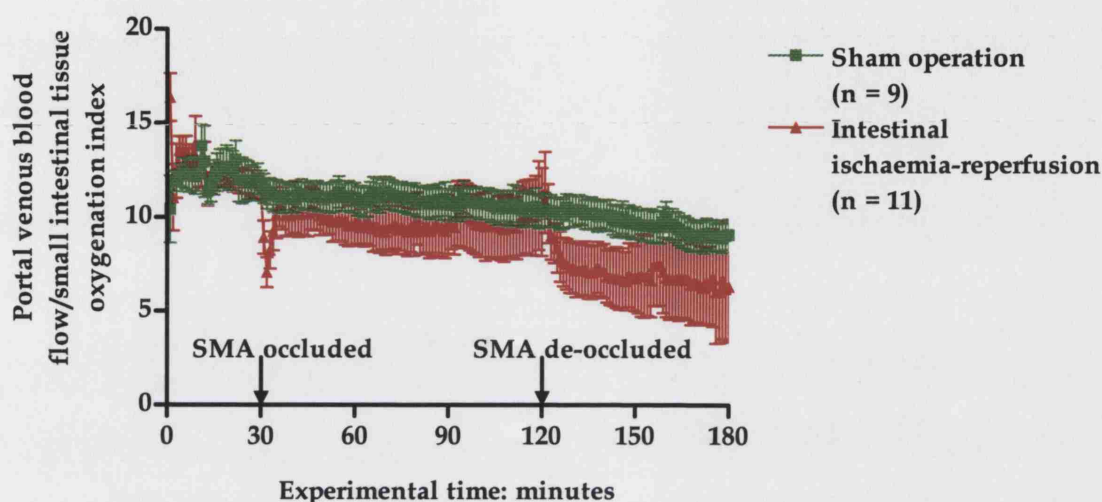


Figure 6.29: Ratio of PVF to small intestinal TOI during sham operation or IIR in adult rats: group mean \pm SEM.

It is clear from these plots that the relationship between SMAF, PVF and small intestinal TOI is not straight-forward. During both run-in and intestinal reperfusion for SMAF, and in all phases of the experiment for PVF, the ratio of flow to TOI follows a similar relationship in both IIR and sham operation groups. However, this ratio decreases with increasing experimental time, an effect that is most clearly seen in PVF/TOI during sham operation. This implies that the relationship between SMAF or PVF and small intestinal TOI alters over the duration of the experiment. This time-dependent effect should be taken into account in the interpretation of this and future work, as well as informing critical review of the literature in this field.

Discussion

This experiment has measured tissue HbO and Hb using NIRS of the intestines of suckling rats in a pilot study, and followed this with a larger study using simultaneous SMAF and PVF transit-time ultrasound measurements, transduced heart rate and arterial blood pressure measurements, and NIRS measurements of small intestinal tissue HbO and Hb concentrations in adult rats during sham operation and IIR.

The changes in intestinal oxygenation suggested by the pilot study reported in experiment 1 using suckling rats implied that intestinal reperfusion was not maintained if reperfusion was prolonged. This has been explored further in the experiments reported in Chapter 4 of this thesis (pages 178 - 226) and is also reported in the literature (Turnage et al, 1996; Nakamura et al, 2001). The demonstration that NIRS of the small intestine may reflect this late failure of intestinal reperfusion was a novel finding. There were technical problems encountered during this pilot study that were addressed before embarking on the second experiment. These related to the commercial NIRS equipment used for the pilot study. This equipment uses a limited algorithm to calculate HbO and Hb, and it is not possible to 'zero' the readings with this equipment to give a true absolute zero for HbO. This resulted in the low readings for TOI recorded in this pilot study. As portal venous blood is approximately 75 -80% saturated, the expected TOI for blood within the intestines ought to lie between 80 and 85%, reflecting the relative contributions of arterial and venous blood. The lack of decline in TOI with intestinal ischaemia is also likely to reflect the poorer data processing algorithms of the NIRS unit used for the pilot study. Because of these technical problems, a switch was made to a custom-built NIR spectrometer for the second experiment with data-processing using customised software designed to address these issues. Despite these technical drawbacks, the pilot study did

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highlight dynamic changes in tissue oxygenation during intestinal reperfusion that were followed up in the second experiment.

The second experiment reported here demonstrated a rise in heart rate in late intestinal reperfusion; this is supported by the literature (Hayward and Lefer, 1999; Khanna et al, 2001) and the findings reported in Chapter 4 of this thesis (pages 178 – 226). The changes in MAP found in this experiment are slightly different from those reported in Chapter 4 of this thesis– the widely reported fall in MAP on intestinal reperfusion (Nakamura et al, 2001; Schmeling et al, 1989) was sustained throughout intestinal reperfusion in this experiment, an effect that was not seen in the experiment reported in Chapter 4 of this thesis. This may have been due to a greater degree of insensible fluid loss in this experiment: it was not possible to approximate the abdominal wall due to the NIRS optode arrangement above the small intestines; therefore the intestines were more exposed and transpiration may have depleted intravascular volume to a greater extent resulting in a more severe haemodynamic consequences of IIR.

Measurements of SMAF and PVF made in this experiment (mean SMAF sham operation 30th minute: 4.06 ± 0.52 ml/min; mean SMAF IIR group: 5.04 ± 0.55 ml/min; mean PVF sham operation 30th minute: 9.73 ± 0.78 ml/min; mean PVF IIR group 30th minute: 9.02 ± 0.58 ml/min) appear to be lower than those reported in the literature - Myers (Myers and Hernandez, 1992) reports a baseline SMAF rate of 18ml/minute in rats – but SMAF and PVF are often reported as flow rates per minute per 100g intestinal tissue, a unit not employed in this experiment. The pattern of flow in the SMA was as predicted – slight fall during run-in, artificially imposed cessation during intestinal ischaemia, then a return to pre-occlusion levels of flow immediately on reperfusion with a gradual decline in flow with prolonged intestinal reperfusion. This finding is echoed in the experiment reported in Chapter 4 of this thesis regarding PVF, and in the literature (Turnage et al, 1996; Nakamura et al, 2001). A broadly similar pattern was seen in PVF, with the

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exception that some flow was maintained (likely by splenic and inferior mesenteric vein contributions to PVF) during SMA occlusion. As hepatic arterial flow was not monitored in this experiment, the 'hepatic arterial buffer response' could not be assessed.

Small intestinal HbO concentration measured by NIRS appeared broadly to follow the patterns established by SMAF and PVF. Small intestinal Hb concentration measurements demonstrated a much larger variability than HbO measurement that is mainly due the derivation of this measurement from a part of the spectrum contributed to by the water signal. When the small intestinal HbO and Hb concentrations were combined to produce the small intestinal TOI, most of this variability was lost as the higher absolute levels of HbO outweighed the variability in the lower absolute levels of deoxy-haemoglobin. In order to produce absolute concentrations of HbO and Hb it was necessary to subject one third of the experimental animals to a brief hypoxic insult in order to convert all the haemoglobin then present in the intestines into Hb. From these readings it was possible to estimate absolute levels of small intestinal Hb and convert relative levels of HbO and Hb measured into absolute concentrations. It is not ideal to have to administer such an insult, but all except one animal was successfully resuscitated, and run-in parameters (heart rate, MAP, SMAF, PVF, small intestinal HbO and Hb) all returned to pre-insult levels before the end of the run-in period.

Overall, a largely homogeneous response was seen in the sham operation group, but the IIR group fell into two types of response. In IIR (A), rats showed a significant fall in SMAF and PVF during intestinal reperfusion that was closely echoed by falls in both small intestinal HbO concentration and TOI. In IIR (B), rats showed a similar fall in SMAF and PVF during intestinal reperfusion, but no concomitant fall in small intestinal HbO concentration or TOI was observed. It is difficult to explain these findings as no direct measurements of tissue oxygenation or high energy metabolites were taken. It may be that IIR (B) animals were able to

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maintain small intestinal HbO concentration and TOI by collateral blood supply from the coeliac axis or inferior mesenteric vein, or that increased oxygen extraction with suppressed oxygen use allowed these animals to maintain tissue oxygenation under conditions of very low arterial inflow. Further experiments to clarify this apparent diversity of response should be aimed at confirming tissue oxygen levels by a different method either directly or via a proxy measure. Alternatively confounding results due to collateral supply could be excluded by ligating intestinal collaterals (Megison et al, 1990).

One of the important aspects of this observation is the dissociation of changes in small intestinal HbO concentration and TOI and arterial inflow into/portal venous efflux from the small intestines. It is clear that the relationship between these parameters is not straightforward in all animals, and this must be taken into account when planning future experiments. These findings are at odds with two experiments reported in the literature which demonstrated positive direct correlations between gastric NIRS and SMA flow in pigs either during haemorrhage (Cohn et al, 2001) or abdominal hypertension with and without haemorrhage and resuscitation (Varela et al, 2001). Their findings are a little surprising as the stomach is not supplied by the SMA. It is also not clear how either of these groups managed the statistical problem of using repeated (and therefore non-independent) values from individual animals to generate correlation data between two variables. In the current experiment, this problem has been addressed by calculating the ratio of SMA and portal venous flow to small intestinal TOI for individual animals for each minute of experimental time, then plotting group means of this ratio for each minute to examine this ratio over time. It is clear from these plots that SMA and portal venous flow within both the sham operation and IIR groups do not have a simple, predictable relationship. The relationship appears to change with experimental time, and – as predicted by the disparity within the IIR group outlined above – the IIR group shows greater ratio

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variability than the sham operation group. The explanation for this complex relationship may lie in changing metabolic activity within the small bowel during the experiment, the presence or absence of collateral flow and changes in haematocrit (and therefore delivery of HbO) during the experiment, as shown in Chapters 3 (pages 145 – 177) and 4 (178 – 226) of this thesis and demonstrated in the literature (Yamamoto et al, 2001).

There are still theoretical problems to be overcome relating to the physics of the passage of near infrared light through tissue of changing physical composition. These theoretical issues include the accurate determination of the path-length of near infrared light within tissue. This is important because the algorithms used to calculate HbO and Hb concentration within tissues rely on a largely unvarying path-length. The use of TOI rather than absolute concentrations side-steps this problem to a certain extent, but does not solve it. Penetration of near infrared light through interposed layers of tissue was not an issue in this experiment as the small bowel was exposed and optodes balanced over it, but would be a problem in translating this technique into a clinical context. One of the advantages of using NIRS on the paediatric intensive care unit is that pre-term and critically sick neonates have thin anterior abdominal walls which are unlikely to contribute a significant barrier to NIRS interrogating the intestines.

Summary and Conclusions

This chapter describes two experiments. The first was a pilot study which established the feasibility of recording HbO and Hb levels from the intestines of suckling rats using NIRS. The second experiment used NIRS, TTUS flow probes and transduction of arterial flow to follow changes in small bowel haemoglobin oxygenation, blood inflow and efflux, and heart rate and MAP during experimentally induced sham operation or IIR in adult rats. A relationship was found between SMAF and PVF and small intestinal TOI that appeared to change with experimental time and experimental conditions.

The measurement of TOI, or more specifically trends in TOI, may be of use in a clinical setting if outstanding theoretical and practical issues are overcome (path-length variation during recordings; absolute zero oxy-haemoglobin determination; penetration/delivery of near infrared light into the abdomen).

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Chapter 7: Summary, final discussion and conclusions

Introduction

This chapter summarises the experiments and findings of this thesis as a whole. Intestinal ischaemia-reperfusion (IIR) injury both locally and affecting the liver was assessed in a rat model. The impact of IIR on the hepatic metabolism in suckling rats was documented, and comparison of this was made to the effect in adult rats; the effect of IIR on blood composition, intestinal and hepatic inflow and haemodynamics, cytokine and endotoxin levels in adult rats at normothermia was compared to the changes seen at hypothermia; and intestinal tissue oxygenation was measured by near infra-red spectroscopy (NIRS) during IIR in adult rats. The impact of age and whole body hypothermia on IIR-related changes was assessed, and possible mechanisms discussed. Finally, future directions for research in this field are suggested.

Summary of thesis

In Chapter 1, the pertinent anatomy, clinical causes, pathophysiology, novel therapeutic strategies and effects of age relating to IIR were discussed. This chapter provided background information and an up-to-date experimental context for the experimental work that followed it.

Experiments described in Chapter 2 used a rat model of IIR (45 or 90 minutes of intestinal ischaemia followed by 60 minutes of intestinal reperfusion) to examine the impact of IIR on the tissue biochemistry of the liver of suckling rats, and compared the changes in hepatic metabolites noted to the changes observed in adult rats. In both suckling and adult rats, the longer intestinal insult resulted in depletion of adenosine triphosphate (ATP) and accumulation of inorganic phosphate (Pi) and alanine. Suckling rats also showed rises in hepatic lactate and

succinate, whereas adult rats showed a drop in hepatic glutamine and higher levels of total hepatic glutathione.

In Chapter 3, experiments to measure Pi levels in the superior mesenteric vein (SMV) and right atrium (RA) after IIR were reported. Pi was initially higher in the SMV early in reperfusion but failed to sustain this rise with prolonged reperfusion. RA Pi increased with prolonged reperfusion to reach a level significantly greater than that following prolonged sham operation, indicating that the liver is able to filter out the Pi load delivered from the reperfused intestines at first, but is unable to sustain this role, and with prolonged reperfusion probably adds to the plasma Pi burden. In a second series of experiments, aortic, SMV and inferior vena cava (IVC) samples were taken during IIR to assess the impact on haematocrit, pH, blood oxygen content and oxygen consumption in the areas drained by the SMV and IVC. IIR resulted in rises in haematocrit and falls in pH at all sites sampled. Blood oxygen content rose in the aorta and SMV, but not in the IVC after IIR. Arterio-venous difference in whole blood oxygen content rose markedly with prolonged reperfusion in the area drained by the IVC only.

Chapter 4 described the monitoring of systemic haemodynamics and hepatic inflow during IIR, and the effect of IIR on blood composition and hepatic metabolites both at normothermia ($37 \pm 0.5^\circ\text{C}$) and hypothermia ($32 \pm 0.5^\circ\text{C}$). Normothermic IIR resulted in tachycardia, decreased final total hepatic inflow (THI), raised haematocrit, reduced pH, reduced hepatic ATP and raised hepatic alanine. Rats that were hypothermic during IIR experienced bradycardia, no significant diminution in final THI, a smaller increase in haematocrit and fall in pH, and no change in hepatic ATP or alanine. The positive correlation between hepatic ATP levels and final THI, and the negative correlation between hepatic alanine levels and final THI seen in normothermic rats after IIR was not seen in hypothermic rats after IIR.

In Chapter 5, the effect of IIR on cytokines (tumour necrosis factor- α [TNF- α] and interleukins 1 β , 6 and 10 [IL-1 β , IL-6, IL-10]) and endotoxin levels in the portal and systemic circulations was examined after normothermic and hypothermic IIR and normothermic sham operation. TNF- α , IL-6 and IL-10 all rose with IIR in normothermic rats but less so in hypothermic rats. Systemic endotoxin levels were lower after hypothermic IIR than after normothermic IIR.

Chapter 6 describes the monitoring of intestinal tissue oxygenation levels during IIR in real-time using NIRS and a rat model. A pilot study using suckling rats was promising, and a full experiment with adult rats showed that changes in intestinal tissue oxygenation were related to changes in superior mesenteric arterial inflow and portal venous outflow during IIR. This relationship changed with experimental time.

Hepatic metabolites after IIR in suckling and adult rats

Hepatic metabolites were measured after 45 or 90 minutes of intestinal ischaemia followed by 60 minutes of intestinal reperfusion, and compared to 105 or 150 minutes of intestinal ischaemia only (IO) or sham operation. The shorter insults in each group did not result in any differential perturbation of the hepatic metabolites measured (except a rise in hepatic adenosine diphosphate [ADP] with IO, and a rise in hepatic glutamine with IO and IIR). The longer series of insults resulted in a fall in hepatic ATP, and rises in hepatic Pi, succinate, lactate and alanine after IIR. This indicates that the shorter insult did not reach a theoretical threshold of intestinal injury required to cause hepatic metabolic perturbation in suckling rats. This threshold may be related to both the duration of intestinal ischaemia and the duration of intestinal reperfusion. It is possible that a more prolonged period of reperfusion after this short ischaemic insult may have resulted in similar hepatic dysfunction to that yielded by the longer insult.

Chapter 7: Summary, final discussion and conclusions

The longer insults delivered to suckling rats were the same as the insults given to adult rats throughout the remainder of the experiments described in this thesis. Adult rats showed a fall in hepatic ATP and glutamine, and a rise in hepatic Pi and alanine after IIR. Overall hepatic concentrations of hepatic Pi, succinate, lactate and alanine were lower, and glutamine and glutathione were higher in adult rats after IIR compared to suckling rats. The rise in hepatic succinate and lactate after IIR in suckling rats indicates either a switch to anaerobic glycolysis in this group – a switch that did not apparently occur in the adult rat group – or an inability to dispose of a higher succinate and lactate load delivered to the liver from the reperfused intestines. The greater apparent sensitivity of suckling rats to the same insult, resulting in anaerobic glycolysis may be related to their smaller circulating volume. Dehydration would have been relatively more severe in the suckling rat group, potentially resulting in greater haemodynamic compromise than that seen in the adult rats. Poorer hepatic perfusion would result in greater tissue hypoxia, and exaggerate the effects of IIR-related injury.

Adult rats experienced a depletion of hepatic glutamine during IIR that was not observed in suckling rats, and adult rat alanine levels did not rise as high as in suckling rats after IIR. This potentially implicates glutamine and alanine as alternative sources of glucose in the adult rat but not, or less so, in the suckling rat. These differences may be due to enzyme pathways availability in the maturing animal liver, or to differential sensitivity of the immature liver to the toxins released by the reperfused intestines. This work should inform the use of glutamine supplementation in critically sick neonates and infants, as relative glutamine depletion after IIR has not been observed in these experiments in suckling rats. Glutamine supplementation in adults is supported by these experiments, if the relative glutamine depletion noted here is functionally important.

The matching fall in hepatic ATP and rise in hepatic Pi after IIR in both suckling and adult rats suggests a 'final common pathway' in hepatic dysfunction after IIR. The disparity in other hepatic metabolite changes after IIR suggests alternative routes to this hepatic energy failure in the mature and immature animal. The greater overall concentration of hepatic glutathione in adult rats may indicate a larger anti-oxidant reserve in adult animals, possibly resulting in the smaller degree of hepatic Pi increase seen in adult rats (potentially less peroxidation of phospholipids, and less cell metabolic disruption and autolysis). Despite the lack of evidence of tissue hypoxia in the adult rat liver, hepatic ATP levels fell, suggesting a toxic effect of IIR on the liver even without local oxygen starvation.

Whole body hypothermia and IIR-related injury

Systemic haemodynamics, hepatic inflow, portal and systemic blood composition, cytokine and endotoxin levels, and hepatic metabolites were measured during and after 90 minutes of intestinal ischaemia and 60 minutes of intestinal reperfusion in adult rats at both normothermia ($37 \pm 0.5^\circ\text{C}$) and hypothermia ($32 \pm 0.5^\circ\text{C}$). IIR at normothermia resulted in profound changes in all of these parameters, but IIR at hypothermia resulted in many of these parameters either changing significantly less or not at all. This implies that whole body hypothermia has a protective effect on the injurious impact of IIR on the whole organism, and may help us to understand part of the mechanism behind this protection.

The reduction in cytokine production by the intestines after hypothermic IIR is likely to have reduced the inflammatory stimulus to the liver and the remainder of the animal. This is supported by a matching reduction in cytokine levels in the systemic samples taken. This reduction in dissemination of pro-inflammatory cytokines, but interestingly a smaller reduction in dissemination of the anti-

inflammatory cytokine measured – interleukin 10 - could have significantly limited the systemic inflammatory response to IIR. This may have reduced the endovascular dysfunction associated with systemic inflammation resulting in less tissue oedema, both locally and systemically. This is supported by the reduction in increase in haematocrit noted after hypothermic IIR both in the splanchnic circulation and systemically. Less endovascular dysfunction may lead to a reduction in altered reactivity in the cardiovascular system – this is supported by the loss of a tachycardic response to prolonged intestinal reperfusion in the hypothermic animals after IIR. Hypothermia may have a direct effect on the cardiovascular system, as these animals became significantly bradycardic after IIR. This was not seen in the hypothermic animals subjected to sham operation, and therefore may be a combined effect of hypothermia and IIR-related toxin release. Endotoxin delivery to the liver was not affected by hypothermia, but systemic endotoxin levels were significantly lower in the hypothermic group. This indicates that the liver is better able to eliminate delivered endotoxin after hypothermic IIR than after normothermic IIR. The reduction in systemic endotoxin release after hypothermic IIR also reduces the dissemination of the IIR-related insult as endotoxin is a potent inflammatory stimulus, and a reduction in endotoxin release will propagate the suppression of pro-inflammatory cytokines outlined above. Hepatic inflow was better maintained after hypothermic IIR than after normothermic IIR. This could have been a consequence of less fluid loss into the interstitium from blood within the intestines, resulting in less viscid blood better able to maintain flow rates. Mean arterial blood pressure was not altered between hypothermic and normothermic IIR groups, so perfusion pressure was not a factor here. Final THI was not less than sham operation in the hypothermic IIR group, but was suppressed in the normothermic IIR group. This implies that hypothermia supported hepatic inflow at expected levels at the very end of the experiment. Maintenance of hepatic inflow may have supported hepatic function

during IIR, resulting in reduced hepatic compromise. This is upheld by the greater concentration of hepatic ATP in the liver after IIR in the hypothermic group, and the lack of rise in hepatic tissue alanine in this group. The correlation between final THI and hepatic ATP (positive) and alanine (negative) concentrations observed in the normothermic IIR group was lost in the hypothermic IIR group, despite a similar range of final THI rates and metabolite concentrations. This indicates that the normothermic IIR group hepatic metabolite levels had a degree of sensitivity to final THI rates, but the hypothermic IIR group did not. Reasons for this might include the lower overall inflammatory influx from the intestines in the latter group, resulting in a lower level of hepatic inflammation and therefore greater ability to withstand the toxic effects of IIR; or a direct suppression of hepatic reactivity to the toxic effects of IIR resulting in less metabolic derangement after an identical insult at normothermia. It is not possible to tease these various effects of hypothermia apart from this experiment.

Monitoring intestinal tissue oxygenation during IIR

NIRS was used to interrogate the intestines during IIR in suckling rats in a pilot study, and results indicated that a full-scale study using adult rats would be worthwhile. NIRS of the intestines, whilst simultaneously monitoring superior mesenteric artery (SMA) inflow and portal venous outflow of blood in real time was performed on adult rats. The tissue oxygen index (TOI) of the intestines changed in a pattern largely predicted by the changes in SMA inflow and portal venous outflow. A plot of the ratio of SMA flow and portal flow to TOI during both IIR and sham operation showed a gradual change in these ratios when plotted over experimental time in both groups, under very different conditions of blood flow and tissue injury. From this it can be deduced that NIRS can be used to produce a TOI from the intestines, and that this TOI has a relationship to the inflow of oxygenated and efflux of deoxygenated blood from the intestines that is

in part time-dependent. This in itself is not surprising, as the transfer of oxygen delivered by the blood to the tissues is a complex, non-linear process dictated by local conditions (pH, temperature, oxygen-haemoglobin dissociation curve). The value of this experiment lies in the demonstration that, although the relationship between arterial inflow and TOI in the intestines is not constant over time, it is relatively unaffected by tissue injury and absolute flow levels. There are significant theoretical problems in the measurement of TOI by NIRS, and these must be overcome before this method of interrogating the intestines can be used to predict and follow intestinal ischaemia clinically.

Future work

This thesis has described differential changes in hepatic metabolites after IIR in suckling and adult rats; the effect of whole body hypothermia on the changes in systemic haemodynamics, hepatic inflow, blood composition, cytokine and endotoxin release and hepatic metabolites after IIR; and the monitoring of intestinal tissue oxygenation during IIR using NIRS. There are at least three directions in which research is worth pursuing using this animal model and extending this work into the clinical sphere.

Firstly, the pathophysiology underlying the IIR insult could be explored in greater detail at the molecular level. Gene expression in tissues both local and remote to the insult may reveal which cytokines are crucial in the dissemination of the inflammatory response to IIR, and which promoter sequences and cell-signalling pathways are used. Also, leukocyte signalling and endovascular excitation could be better understood leading to a clearer picture of local interactions and the response of remote organs to IIR. These strategies may reveal attack points for the future treatment of IIR in both the experimental and, eventually, the clinical setting. The effects of age on these responses may allow therapies tailored to the age of the patients involved.

Secondly, the use of whole body hypothermia is worth pursuing as the benefits demonstrated in this thesis are so wide-ranging. It would be crucial to explore the effect of whole-body hypothermia applied only in the reperfusion phase of IIR-injury as this is the phase most likely to be encountered clinically at the point of intervention. An alternative approach could be the development of regional hypothermia – possibly of the intestines alone or the intestines and liver – and comparison of the benefit of this to whole body hypothermia. As a coda to the molecular work proposed above, exploration of the molecular basis of the protective effect of whole body hypothermia may reveal alternative strategies producing the same effect without the need for a global drop in core temperature. A candidate for the molecular basis of the benefit of hypothermia is the heat shock response but studies to date have shown mixed results, as discussed in Chapter 1 of this thesis (pages 73 – 76). It is possible that hypothermia could eventually form the basis of a clinical trial of treatment of IIR-induced injury. This may possibly involve the treatment of neonates with necrotizing enterocolitis or patients after revascularisation following acute mesenteric ischaemia (both groups having IIR-related injuries and likely to require intensive care during the recovery phase, making therapeutic intervention of this kind more feasible).

Finally, the use of NIRS to interrogate the intestines during IIR could result in the development of a useful clinical tool to guide diagnosis and ongoing success of treatment of IIR-injury. It has been indicated in this thesis how difficult the diagnosis of IIR may be, and an accurate, inexpensive, non-invasive, continuous method, not using ionising radiation would be a major advance. More work on the technical issues pertaining to this method, and further animal work relating intestinal tissue oxygenation index to tissue viability may yield an exciting new area of clinical monitoring.

Summary and Conclusions

The experiments described in this thesis demonstrate that intestinal ischaemia-reperfusion perturbs hepatic metabolism in suckling and adult rats in subtly different ways. Intestinal ischaemia-reperfusion results in a series of changes in systemic haemodynamics, hepatic inflow, blood composition, cytokine and endotoxin release, and hepatic metabolites that are abrogated by whole-body hypothermia. It is possible to follow changes in intestinal tissue oxygenation in real-time using near infra-red spectroscopy, but the relationship of intestinal tissue oxygenation to arterial inflow into and venous efflux out of the intestines is complex. It is possible to envisage many further studies in this field, including a more detailed examination of the molecular mechanisms underlying both IIR-injury and the benefits of whole-body hypothermia, and the further development of near infra-red spectroscopy of the intestines. It is a wholly admissible fact that observations made in an animal model under general anaesthetic may not directly pertain to the clinical situation, but it is hoped that this body of work may lead to further insights into the diagnosis, consequences and treatment of conditions characterised by intestinal ischaemia-reperfusion injury.